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*OCCURRENCE OF URINARY ACID MUCOPOLYSACCHARIDES IN THE HURLER SYNDROME**

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Communicated by Lowell T. Coggeshall, April 3, 1957

The presence of abnormal amounts of a chemically unidentified substance in tissues of patients with the Hurler syndrome (lipochondrodystrophy gargoylism dysostosis multiplex) has been recognized.[1] Brante[2] recovered a substance with characteristics of an acid mucopolysaccharide from tissues obtained at autopsy (liver, meninges) of patients with this disorder. He suggested that this material, which was metachromatic and contained 3.9 per cent sulfur, 27 per cent hexosamine, and 26 per cent glucuronic acid, was similar to or identical with chondroitinsulfuric acid. Dawson[3] and Uzman[4] have also suggested that the "storage material" in affected tissues is a complex polysaccharide. Since acid mucopolysaccharides are not known to be synthesized in the parenchymal cells affected in this disease (e.g., liver, spleen, nervous system) it seemed possible that widespread deposition in tissues results from an increase in circulating mucopolysaccharides. Accordingly, an attempt was made to determine whether urine of afflicted individuals contains unusual amounts or types of mucopolysaccharides. This communication describes the isolation and identification of two acid mucopolysaccharides from the urine or a six-year-old Negro girl who has the typical grotesque skeletal and facial deformities, hepatosplenomegaly, and corneal clouding characteristic of this disorder. Preliminary studies on a second, male, patient with the Hurler syndrome indicated the presence of similar materials.

Experimental.—After dialysis against cold running tap water, 48-hour urine samples containing thymol were concentrated *in vacuo* at 50° C. to $^1/_{10}$ volume. Sodium hydroxide was added to make a 2 per cent solution, and, after 24 hours at room temperature, dialysis against cold running tap water was repeated. Ten milligrams of one-time crystallized trypsin was added and the mixture dialyzed against 0.1 *M*, pH 8.0, phosphate buffer at 38° C., with external stirring for 4–5 days. Protein was precipitated by the addition of $^1/_2$ volume of 40 per cent trichloroacetic acid and was removed by centrifugation after standing at 4° C. for 1 hour. After dialysis against distilled water, the solution was concentrated to approximately $^1/_{10}$ volume, and the "crude mucopolysaccharide" fraction was precipitated by the addition of 4 volumes of 95 per cent ethanol in the presence of 2 per cent sodium acetate. The precipitate which formed after 48 hours at 4° C.

was collected by centrifugation, washed with absolute ethanol and ether, and dried *in vacuo* in a desiccator containing calcium chloride and paraffin.

These "crude mucopolysaccharide" fractions were purified by slab electrophoresis as described by Schiller *et al.*[5] One hundred and fifty to 200 mg. of "crude mucopolysaccharide" per 48-hour sample was isolated from the urine of this patient, as compared with 20–50 mg. per 48-hour sample obtained from the urine of normal individuals of comparable age and sex. The "crude mucopolysaccharide" obtained from normals, when subjected to slab electrophoresis, yielded insufficient material for further identification. Recently, Di Ferrante and Rich[6] isolated from 23 liters of pooled normal human urine 140 mg. of an acid mucopolysaccharide which was indistinguishable from chondroitinsulfuric acid–A (CSA-A).

Slab electrophoresis of the "crude mucopolysaccharide" fraction derived from the urine of the patient with the Hurler syndrome indicated two peaks. Most of the material was present in the rapidly migrating peak, which had a mobility comparable to that observed for CSA-A and chondroitinsulfuric acid–B (CSA-B, β-heparin).[5] Similarity of behavior to CSA-A and CSA-B was also noted by paper electrophoresis, with respect both to mobility and to metachromatic staining with toluidine blue.

The analyses (Table 1) of the more rapidly migrating peak are consistent with an

TABLE 1

COMPOSITION OF MUCOPOLYSACCHARIDES ISOLATED FROM URINE*

	Hexosamine†	Nitrogen‡	Uronic Acid§	Ester Sulfur‖	N-Acetyl#
"Crude fraction," normal	1.00	3.42	0.71	...	...
"Crude fraction," Hurler syndrome	1.00	2.30	0.76	...	...
"Purified fraction," rapidly migrating peak, Hurler syndrome	1.00	1.32	0.55	1.22	1.37
"Purified fraction," slowly migrating peak, Hurler syndrome	1.00	1.46	1.40	1.17	0.87

* All analyses on each fraction were performed on the same solution, with the exception of the N-acetyl determination on the rapidly migrating peak. Analyses are expressed as molar ratios with respect to hexosamine.

† Hexosamine was determined by the method of Boas (*J. Biol. Chem.*, **204**, 553, 1953), omitting the Dowex treatment.

‡ Nitrogen was determined by a micro-Kjeldahl procedure.

§ Uronic acid was determined by the method of Dische (*J. Biol. Chem.*, **167**, 189, 1947)

‖ Ester sulfur was determined by the method of L. Anderson (*Acta Chem. Scand.*, **7**, 689, 1953).

N-acetyl determination by chromic oxidation according to the procedure of E. Wiesenberger (*Mikrochim. Acta*, **33**, 51, 1948).

acid mucopolysaccharide containing one sulfate group per disaccharide repeating unit. The values for hexosamine by the method used[7] are generally about 10 per cent low. Some contamination by protein is present, as indicated by the slightly elevated nitrogen value and by the demonstration of free amino acids in hydrolyzates subjected to paper chromatography. The chromatograms were developed with tertiary amyl alcohol–formic acid–water (8:2:3), and the amino acids were demonstrated by the ninhydrin color reaction.

The low uronic acid value obtained by the carbazole method[8] is characteristic of CSA-B.[5] As has been found for CSA-B,[5,9] this material is resistant to the action of testicular hyaluronidase, as demonstrated by the method of Mathews, Roseman, and Dorfman.[10] The optical rotation was $[\alpha]_D^{25} = -68°$, calculated on the basis of hexosamine content of the solution. Galactosamine was demonstrated by the method of Stoffyn and Jeanloz.[11]

CSA-B has been isolated previously from skin,[5, 12] gastric mucosa,[13] and beef lung.[14] It differs from CSA-A in that it contains L-iduronic acid[15, 16] instead of D-glucuronic acid. The presence of L-iduronic acid in this material has been demonstrated by paper chromatography following mild acid hydrolysis.[15] The absence of sialic acid was indicated by a negative direct Ehrlich reaction.[17] Preliminary studies[18] indicate that this material has antithrombic activity similar to that of CSA-B[19] obtained from beef lung.

The more slowly migrating peak had an electrophoretic mobility somewhat less than CSA-A and CSA-B but greater than that of hyaluronic acid. Analyses of this material (Table 1) were consistent with an acid mucopolysaccharide containing one sulfate group per disaccharide unit. The high uronic acid value obtained by the carbazole method is of interest in view of the known high values which are obtained with heparin.[8] This material was also resistant to the action of testicular hyaluronidase. Glucosamine was demonstrated by the method of Stoffyn and Jeanloz.[11] The optical rotation was $[\alpha]_D^{20} = +39°$, based on hexosamine content. These data suggest that this substance is similar to that isolated by Jorpes and Gardell[20] from ox liver, which they named "heparin monosulfuric acid." Meyer[21] has recently indicated the existence of a similar substance in aorta and amyloid.

Discussion.—The data presented indicate that a marked quantitative deviation in the excretion of acid mucopolysaccharides occurs in the Hurler syndrome. Although the two mucopolysaccharides identified occur in normal connective tissues, they have not been previously isolated from urine. Available evidence indicates that normal urine contains only small amounts of CSA-A, in contrast to the finding in the Hurler syndrome of relatively large amounts of CSA-B (β-heparin) and small amounts of a second mucopolysaccharide, tentatively identified as heparin monosulfuric acid.

The site of origin of these polysaccharides is unknown, but it is unlikely that they are formed in all of the parenchymal cells in which abnormal accumulation occurs. More reasonable is the assumption that the fundamental defect resides in the connective tissues. The nature of this defect cannot be ascertained in the absence of more adequate knowledge of the metabolism of these mucopolysaccharides. In any case, it is probable that the distortion of tissues in this heritable disorder is related to an abnormality of mucopolysaccharide metabolism. The Hurler syndrome thus may be considered the first known example of a human disorder involving such a metabolic anomaly.

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† Postdoctoral Fellow of the Arthritis and Rheumatism Foundation.

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ASYMMETRIC DISTRIBUTION OF C^{14} IN SUGARS FORMED DURING PHOTOSYNTHESIS

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Communicated by D. D. van Slyke, March 12, 1957

The concept of the conversion of $C^{14}O_2$ to labeled sugars during photosynthesis as reported by Calvin and coworkers[1, 2] is the following: (1) carbon dioxide fixation involving the carboxylation of a symmetrically labeled two-carbon piece derived from ribulose diphosphate to yield phosphoglyceric acid predominantly labeled in the carboxyl carbon (CH_2*OPO_3H_2-C*HOH-C**OOH), (2) a cyclic transketolase-transaldolase sequence involving fructose-6-phosphate, sedoheptulose-7-phosphate, and pentulose-5-phosphate to produce the symmetrically labeled CO_2 acceptor, and (3) conversion of the phosphoglyceric acid via the Embden-Meyerhof sequence to yield a hexose sugar predominantly labeled in carbon atoms 3, 4 (C-3, C-4) with carbon atoms 1, 6 equal to 2, 5 (C*-C*-C**-C**-C*-C*).[3–6] In earlier papers the hexose degradation data were obtained by the *Lactobacillus casei*[7] procedure, which yields pairs of carbon atoms. In the present report, we have investigated the distribution of tracer in sugars produced during photosynthesis in $C^{14}O_2$ using the *Leuconostoc mesenteroides*[8, 9] degradation method, which permits a determination of the C^{14} content of the individual carbon atoms. The results of these experiments and their implications for the pathway of carbon dioxide to carbohydrate during photosynthesis are reported in this communication.

Methods and Materials.—The *Chlorella* was grown as described by Kandler,[10] except that the light source was fluorescent lamps with an intensity of approximately 1,000 foot-candles. The organisms were harvested by centrifugation, washed twice with water, and suspended in distilled water or potassium phosphate buffer. The tobacco, sunflower, and Canna leaves, including petioles, were removed from mature greenhouse stock plants.

The 10- and 60-second experiments with algae were carried out in a "lollipop"

apparatus, as described by Calvin.[1] Conventional Warburg manometry techniques were employed for the longer-time algal experiments involving the effect of glucose on photosynthesis. In the leaf experiments, the photosynthesis apparatus was a desiccator type of chamber. In all experiments the light source was Sylvania Birdseye Floodlites (150 watts, 120 volts).

In general, the photosynthesis was terminated by immersing the test substances in boiling 80 per cent ethanol. The 80 per cent ethanol extract and two 20 per cent ethanol washings were concentrated under vacuum to approximately 1 ml. The residue was analyzed by two-dimensional paper chromatography (solvents: water-saturated phenol and butanol–propionic acid; paper: oxalic acid–washed Whatman No. 4). The area of the chromatogram occupied by the sucrose was eluted, and the eluate was hydrolyzed by means of invertase and rechromatographed using water-saturated phenol as solvent to separate the monosaccharides. The residue remaining after the 80 per cent ethanol extraction was treated twice with cold 20 per cent ethanol, then washed several times with hot water.

Following the washing, the starch glucose was derived by hydrolyzing the cells with 1 *N* HCl for 45 minutes. Paper chromatography indicated that essentially all the tracer resided in glucose. The hydrolyzate was brought to pH 6–7 with 1 *N* KOH.

Glucose was degraded by fermentation with *Leuconostoc mesenteroides*, which has been shown by Gunsalus and Gibbs to produce CO_2 from carbon atom 1, ethanol from carbon atoms 2 and 3, and lactate from carbon atoms 4, 5, and 6. The ethanol was oxidized to acetic acid by heating for 2 hours at 90° C. with 0.5 gm. of potassium dichromate in 4 *N* sulfuric acid. The activity of the carboxyl carbon of the acetic acid was obtained by the method of Phares. Methyl carbon activity was determined by difference between the activity of the whole acetic acid molecule obtained by a persulfate oxidation and the carboxyl carbon determination. Lactate was subjected to an oxidation with chromium trioxide to yield CO_2 and acetic acid. In order to avoid the addition of carrier ethanol or lactate, about 400–500 μmoles of glucose were fermented.

Radioactivity measurements were made with a Bernstein-Ballentein methane flow counter. All samples were converted to barium carbonate and counted at infinite thickness. Activity is expressed in Tables 1–3 as millimicrocuries per milligram of carbon (mμC/mgC).

Results and Discussion.—All the glucoses formed during the short periods of photosynthesis in $C^{14}O_2$ (Table 1) possess an asymmetrical distribution of C^{14}. The ratio of carbon atom 3 to carbon atom 1 (C-1, aldehyde carbon) and/or C-2 is less than that of C-4 to C-5 and/or C-6. This type of labeling pattern in the glucose moiety of sucrose and starch is similar to that reported in our earlier communication[11] for the glucose phosphate esters (monophosphate, uridine diphosphate glucose, and an unidentified glucose phosphate).

The labeling pattern of the three glucose phosphate esters of our previous communication can be compared with the data tabulated in the first line of Table 1, since the four glucose units were derived from the same experiment. An average of the four glucose degradations in which the activity of the various carbon atoms are expressed in percentage of the total glucose molecule is as follows: C-1(7.9), C-2(7.6), C-3(33.8), C-4(42.5), C-5(3.5), C-6(4.7).

TABLE 1

DISTRIBUTION OF C^{14} IN GLUCOSE

PLANT	LIGHT INTENSITY (FOOT-CANDLES)	TIME	GLUCOSE SOURCE	TRACER CONTENT OF GLUCOSE CARBON ATOMS ($M\mu C/mgC$) 1	2	3	4	5	6
*Chlorella**	4,000	10 sec.	Starch	0.35	0.27	3.67	4.90	0.10	0.16
Chlorella†	4,000	60 sec.	Starch	1.16	1.15	5.16	7.00	0.42	0.46
Chlorella‡	700	45 min.	Starch	22.5	22.8	25.4	26.4	22.5	23.3
Tobacco§	4,000	50 sec.	Starch	2.69	4.30	11.0	18.6	1.17	2.99
Tobacco§	100	180 sec.	Starch	8.55	10.7	25.9	37.5	9.12	8.21
Sunflower§	70	15 min.	Sucrose	0.55	0.60	1.20	2.29	0.48	0.54
Canna	2,000	24 hrs.	Sucrose	5.36	5.16	5.19	5.08	5.08	5.12

* Thirty milliliters of a *Chlorella* suspension (1.5 ml. packed cells per 100 ml. water) was illuminated 5 minutes in a nitrogen atmosphere before the introduction of 10 μmoles of $NaHC^{14}O_3$ containing 135 microcuries.

† Thirty milliliters of a *Chlorella* suspension (1.5 ml. packed cells per 100 ml. water) was incubated with 10 μmoles of $NaHC^{14}O_3$, containing 135 microcuries, in the dark for 5 minutes before photosynthesis occurred.

‡ Five milliliters of *Chlorella* suspension (1.0 ml. packed cells per 100 ml. *M*/30 potassium phosphate buffer, pH 5.6) was illuminated in a Warburg vessel. Introduction of $NaHC^{14}O_3$ (50 μmoles, 40 microcuries) at same time light was applied.

§ The leaves were illuminated 5 minutes in an air atmosphere before the introduction of 10 μmoles of $C^{14}O_2$ containing approximately 100 microcuries.

TABLE 2*

EFFECT OF GLUCOSE FEEDING ON POSITION LABELING IN GLUCOSE OF SUCROSE FORMED DURING PHOTOSYNTHESIS IN $C^{14}O_2$

TIME (MINUTES)	CO_2 (μMOLES)	TRACER CONTENT OF GLUCOSE CARBON ATOMS ($M\mu C/mgC$) WITHOUT GLUCOSE 1	2	3	4	5	6	WITH GLUCOSE 1	2	3	4	5	6
15	10	3.16	2.92	4.00	5.21	1.25	1.67	8.08	8.34	13.3	13.8	2.46	2.09
45	50	2.49	2.16	2.52	3.52	1.12	1.46	8.42	8.33	14.9	18.3	3.58	4.42
180	200	4.34	4.31	4.25	5.12	3.98	4.36	3.39	2.53	4.92	6.23	1.30	1.55

* The *Chlorella* were starved by shaking the cells in water at room temperature for 16 hours in the dark. After centrifuging, they were suspended in *M*/30 potassium phosphate buffer, pH 5.6 (1.0 ml. of packed cells per 100 ml. buffer). The experiment was carried out in six 150-ml. Warburg vessels, one set of three containing 5 ml. of the cell suspension, the other, 5 ml. of suspension containing 55 μmoles/ml. glucose. The side arms carried either 10, 50, or 200 μmoles of $NaHC^{14}O_3$ containing 2.75 microcuries. After the vessels were shaken 10 minutes in the dark, the $NaHC^{14}O_3$ was tipped in and illumination was begun. An illumination of 700 foot-candles was used, which resulted in glucose respiration (5-fold over endogenous) and photosynthesis being at the compensation point. After the appropriate time, the contents of the vessels were pipetted into cold centrifuge tubes containing ice water. After the cells were centrifuged and the supernatant fluid discarded, they were extracted twice with hot water. The glucose moiety of the sucrose was isolated for degradation from the hot-water washings, as described under "Methods and Materials." The starch glucose was obtained for degradation as described under "Methods and Materials."

TABLE 3*

EFFECT OF GLUCOSE FEEDING ON POSITION LABELING IN STARCH FORMED DURING PHOTOSYNTHESIS IN $C^{14}O_2$

TIME (MINUTES)	CO_2 (μMOLES)	TRACER CONTENT OF GLUCOSE CARBON ATOMS ($M\mu C/mgC$) Without Glucose 1	2	3	4	5	6	With Glucose 1	2	3	4	5	6
15	10	1.03	0.71	1.71	2.08	0.37	0.53	1.61	1.59	2.88	3.08	0.60	0.82
45	50	..	..	..	..	..	..	3.00	2.93	5.88	7.15	1.52	1.57
180	200	2.21	2.32	2.42	2.83	2.31	2.11	4.45	3.60	8.42	10.42	2.44	2.64

* See note to Table 2.

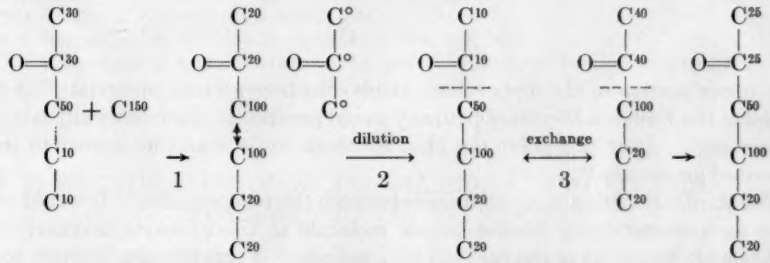
Our earlier communication also reported degradation data for two hexose phosphate esters corresponding to the glucose data listed in the second line of Table 1. The average values in percentage of the total glucose molecule for the three degradations are C-1(5.5), C-2(5.6), C-3(41.0), C-4(46.0), C-5(0.8), C-6(1.2).

The isotope-distribution data recorded in Table 1 were obtained with *Chlorella* and leaves permitted to photosynthesize in $C^{14}O_2$ for short time periods. Since the findings of Kandler[12] and Simonis and Grube[13] have indicated that exogenous glucose may influence the photosynthetic process, an experiment was carried out in which the rate of glucose respiration and photosynthesis were at the compensa-

tion point, with the desire that the exogenous C^{12}-glucose would enter the photosynthetic cycle, dilute the compound or compounds which are closely connected with $C^{14}O_2$ reduction, and thereby prolong the period of asymmetric distribution of label in the newly formed carbohydrate. The isotope-distribution data tabulated in Tables 2 and 3 strongly indicate that a substrate of respiration can, at least at the compensation point, enter the photosynthetic cycle and be converted into compounds involved in the pathway between carbon dioxide and the hexose molecule. Thus, even following a photosynthesis period of 180 minutes, the *Chlorella* which received an exogenous C^{12}-glucose supply still possessed sucrose and starch whose glucose moieties exhibited a marked asymmetry in isotope distribution, whereas the control algae (minus glucose) contained a uniformly labeled hexose unit.

At present, three mechanisms can be suggested to account for a glucose molecule possessing a labeling pattern in which the ratio of C-3 to C-1 and/or C-2 is less than that of C-4 to C-5 and/or C-6.

One pathway, which was suggested to us by Dr. Paul Marks, we have titled the ' exchange and dilution pathway." This "pathway" involves a slight modification of the photosynthetic cycle published recently by Bassham, Barker, Calvin, and Quarck.[14] It may be summarized in the following manner (the values assigned to the carbon atoms of the various compounds are arbitrary and are expressed as per cent of total activity; they are based on the data of Calvin and co-workers):



1. The conversion of the aymmetrically labeled ribulose diphosphate and carbon dioxide into two moles of phosphoglyceric acid, followed by a conversion to a mole of glyceraldehyde-3-phosphate and one of dihydroxyacetone phosphate catalyzed by the carboxylating enzyme, phosphoglyceric acid transphosphorylase, triose phosphate dehydrogenase, and triose phosphate isomerase. Carbon dioxide has been assigned the highest value, since the ribulose diphosphate has presumably been diluted by endogenous material.

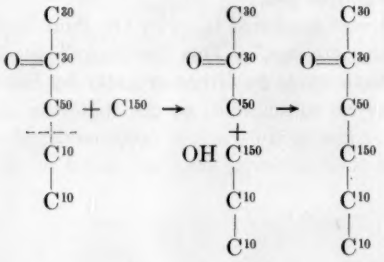
2. Dilution of the isotopic dihydroxyacetone phosphate by a pool of inactive dihydroxyacetone phosphate derived from the breakdown of endogenous fat. Dilution prior to condensation would produce an asymmetrically labeled fructose phosphate molecule.

3. A rapid exchange catalyzed by transketolase between the "active glycolaldehyde" moieties of fructose-6-phosphate and pentulose phosphate. The result of the exchange is an increase in tracer C-1 and C-2 of the hexose phosphate molecule. Thus a combination of dilution by endogenous dihydroxyacetone phosphate and

an exchange catalyzed by transketolase could produce a hexose unit whose isotope distribution is in accord with the obtained data. The weakest point in this hypothesis is the source of dihydroxyacetone phosphate in the starved algal cells. An investigation involving a kinetic study on the specific activity of the various intermediates would shed light on this point.

A second pathway for forming an asymmetrically labeled hexose molecule is based principally on our observation that C-4 has the highest activity of the hexose carbon atoms. Since the $C^{14}O_2$ has the highest specific activity of all components of the photosynthetic reactions, a direct conversion of the CO_2 to C-4 of the hexose unit would keep this carbon atom of the sugar molecule with the highest specific activity until a uniformly labeled molecule was synthesized.

The hypothesis is proposed that pentulose diphosphate is cleaved between C-3 and C-4. The diose phosphate piece (C-4 and C-5) is condensed with a reduced form of CO_2 to yield glyceraldehyde-3-phosphate:



The upper portion of the diphosphate yields dihydroxyacetone phosphate. A reversal of the Embden-Meyerhof pathway would produce an asymmetrically labeled hexose unit. After this point the photosynthetic cycle would be similar to that described previously.[13]

The third mechanism is a compromise between the two proposals. It would produce an asymmetrically labeled hexose molecule in the following manner: (1) addition of CO_2 to C-4 of the pentose, (2) a reduction of this primary addition compound by the reducing power generated by light, (3) a rearrangement in which the new carbon atom becomes C-4 of the hexose, and (4) a lack of reducing power resulting in the primary addition product being split into two molecules of PGA.

It is evident that the basic difference between the three schemes is the fate of the carbon dioxide in the initial steps. The first and third proposes a carboxylation catalyzed by the ribulose diphosphate carboxylating enzyme, followed by a reduction either as a whole unit or after splitting into two molecules of PGA, while the second speculates on a direct reduction of the CO_2 subsequent to an addition. Further studies will be required to elucidate the significance of this asymmetric type of label in the hexose molecule.

Summary.—Photosynthesis was carried out with *Chlorella* and the leaves of higher plants using $C^{14}O_2$ under various conditions. The glucose isolated from starch and sucrose produced during short periods of photosynthesis possessed an asymmetric distribution of tracer. This pattern of label was also obtained during longer periods of photosynthesis in the presence of a supply of exogenous glucose.

The distribution of tracer is not that which is expected if two equal triose phosphates combined to yield a hexose phosphate.

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*MULTIPLICATION OF ANIMAL CELLS IN SUSPENSION MEASURED BY COLONY COUNTS**

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During the past few years the development of new techniques for the cultivation of animal cells in vitro has facilitated the quantitative study of many aspects of cell biology. At present the most commonly used method of propagating cell strains is based on the ability of cells to multiply while attached to a glass surface. The cells may be subcultured by removing them from the surface into suspension and then distributing them into other vessels, where they will again adhere to the glass and populate the surface. This procedure has been developed by Earle and his associates[1, 2] into the so-called quantitative replicate culture technique and applied to a variety of studies with animal cells. Despite the technical advance represented by this method, there are, nevertheless, serious experimental limitations

inherent in the use of glass surfaces for cultivating large cell populations. Perhaps the most obvious of these is the problem of removing representative samples at will during the growth of a cell population. In addition, subculture requires the removal of cells from the surface, with consequent risk of cell injury.

In order to obviate these restrictions, efforts have been made by several investigators to propagate animal cells in suspension.[3-5] Previous communications from this laboratory have described a technique that accomplishes this purpose.[6, 7] It was shown that, for several strains, the growth of animal cell populations in suspension, estimated by enumeration of cells in a counting chamber, is similar to that of bacteria, in that lag, logarithmic, and stationary phases were observed. Cells could be kept continuously in the logarithmic phase of multiplication by subculturing at frequent intervals.

While such a system would seem to be ideally suited to biochemical studies, its full usefulness cannot be realized without some knowledge of the number of viable cells in the cultures. To obtain this information, we have used the clone technique recently developed by Puck and his associates.[8, 9] In the present paper it will be shown that cells grown in suspension readily form colonies on a glass surface and that, for two strains at least, they will plate with a high and constant efficiency during the logarithmic phase of growth.

Two strains of cells were used in this work: (1) Earle's L strain, a clone derived originally from a culture of adult mouse connective-tissue cells,[10] and (2) a clone, selected in this laboratory, from an altered monkey kidney cell population provided by Dr. R. C. Parker.[11] The method for cultivating cells in suspension, and the medium, have been described in detail elsewhere.[7] In brief, large test tubes containing the cell suspensions were rotated at 60 rpm around their long axes at 37° C. The medium contained 20 per cent horse serum in solution CMRL-1066,[11] which is a defined mixture of salts, amino acids, vitamins, coenzymes, and other metabolites. Cells of both strains multiplied readily under these conditions, with generation times of 20–25 hours in the logarithmic phase of growth. Stock cultures were kept continuously in the logarithmic phase by frequent subculture.

When cells from a logarithmic-phase culture were placed in a stationary container, macroscopically visible colonies appeared on the glass surface after 6–10 days' incubation. A linear relationship was obtained between the number of cells plated (10–500) and the number of colonies formed. The procedure for plating the cells was similar to that used by Puck, Marcus, and Cieciura[9] for suspensions of cells from trypsin-treated monolayers. For the L strain, daily enumeration of the cells in ten randomly selected colonies showed that multiplication of the individual cells began 12 hours after plating and continued logarithmically, with a doubling time of 18 hours, until the colonial populations reached 500 cells or more. Since we found in these experiments that both the plating efficiency, i.e., the percentage of plated cells that developed into colonies, and the rate of cell increase within colonies varied greatly with the particular lot of horse serum employed, a selected serum was used in the remainder of the work.

In order to determine the increase in a cell population by the colony-count method a rotating culture containing about 5×10^4 cells per milliliter was prepared from a logarithmic-phase stock suspension. At intervals samples were removed and the cell densities estimated with a hemocytometer.[12] Serial dilutions of the sample

were made in solution 1066 at room temperature. One hundred cells were plated in each of six Petri dishes (6-cm. diameter) containing 5 ml. of complete medium (solution 1066 supplemented with horse serum) previously equilibrated at 37° C. in an atmosphere of 5 per cent CO_2. After incubation of the plates at 37° C. for 8–10 days in the 5 per cent CO_2 atmosphere, the colonies were washed with buffered saline, fixed with Bouin's solution, treated with Giemsa's stain, and scored. The colonies of L cells on a representative plate are shown in Figure 1.

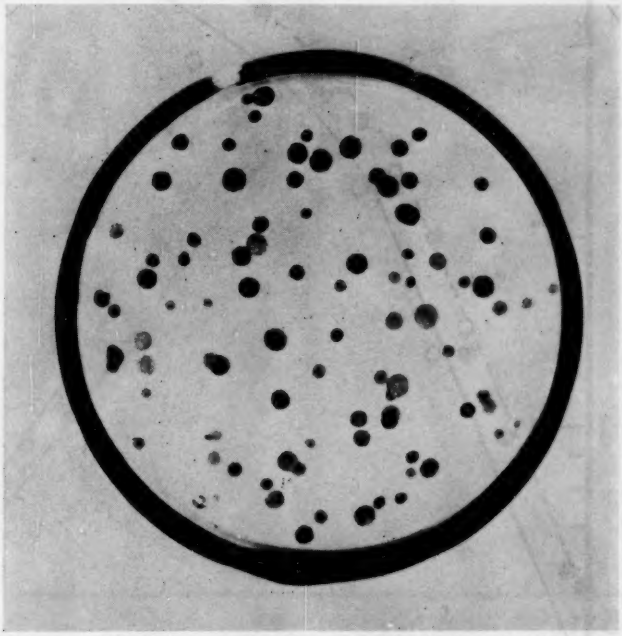

FIG. 1.—Colonies of L-strain cells obtained by plating 100 cells from a suspension in the logarithmic phase of multiplication. The colonies were fixed and treated with Giemsa's stain.

Figure 2 shows the growth curves obtained for L cells by hemocytometer count (*upper curve*) and by plate count (*lower curve*). Each point on the lower curve represents the average of six plates, or 400–600 colonies. The method of least squares was used to fit the exponential portion of this curve and indicated that the standard deviation of the points from the line was about 12 per cent. A similar set of curves is shown in Figure 2 for a suspension of the monkey kidney cells. The average plate counts deviate from the linear part of the curve by 8 per cent. The errors, which include day-to-day variation and sampling errors, are comparable to those obtained in colony counts with bacteria.

It will be seen that the plating efficiencies in both experiments remained constant at about 75 per cent throughout the logarithmic phase of growth. During the stationary phase, however, the plating efficiencies may decrease significantly (Fig. 3). This decrease was very marked in some other experiments, indicating that the cells may undergo an extensive physiological change during this period.

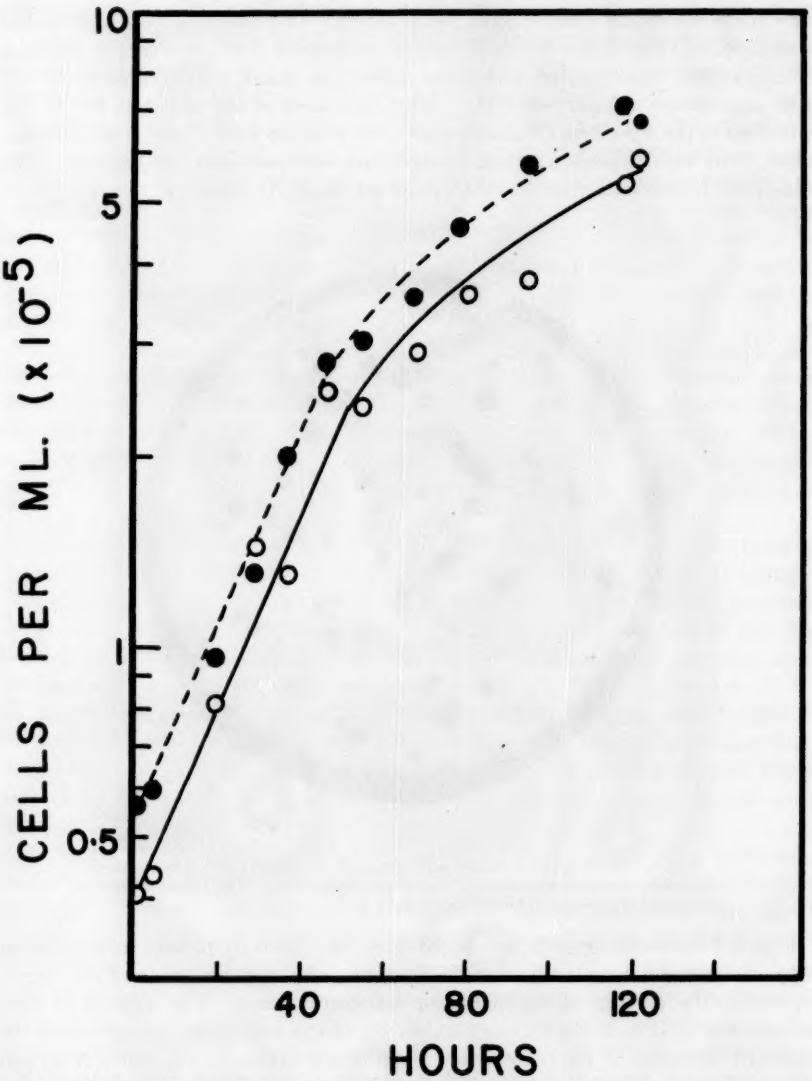

FIG. 2.—Multiplication of L-strain cells in suspension. *Upper curve:* hemocytometer count; *lower curve:* colony count.

It is of interest now to inquire whether the 75 per cent plating efficiency observed during logarithmic growth represents all the viable cells in the population or whether some inadequacy in the plating procedure or in the medium permits only 75 per cent of the cells to form colonies. In an effort to answer this question, we have utilized the feeder-layer technique developed by Puck *et al.*[8, 9] Table 1 shows the results of several experiments in which monkey kidney cells from loga-

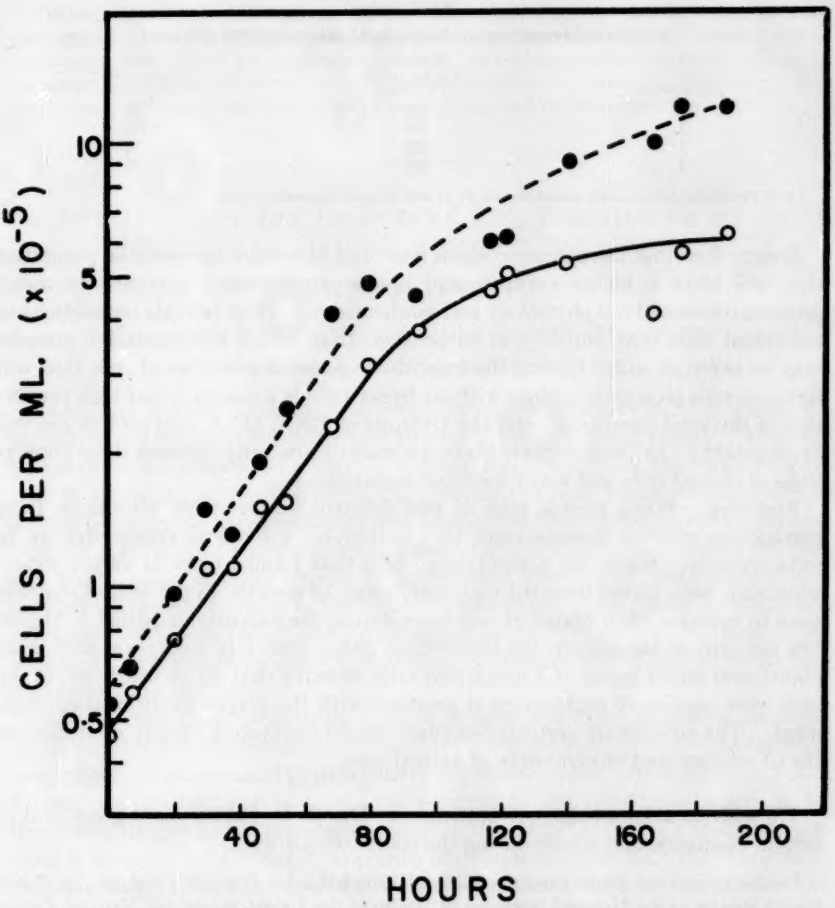

FIG. 3.—Multiplication of altered monkey kidney cells in suspension. *Upper curve:* hemocytometer count; *lower curve:* colony count.

rithmic-phase suspensions were plated over feeder layers of giant cells obtained from X-irradiated cultures of the same cell strain. In each case the plating efficiency was higher in the presence of feeder cells than in their absence and was frequently in the neighborhood of 100 per cent. These observations indicate that, during the logarithmic phase of multiplication, nearly all the cells possess the ability to form colonies under the improved plating conditions provided by the presence of feeder cells. It should be mentioned, however, that for many practical purposes, e.g., studies on virus and radiation sensitivity of cells during the period of logarithmic increase, a plating efficiency of 75 per cent achieved without the use of feeder cells is adequate.

TABLE 1

PLATING EFFICIENCY OF ALTERED MONKEY KIDNEY CELLS

Experiment	Without Feeder Cells (Per Cent)	With Feeder Cells* (Per Cent)
1	58	98
2	58	92
3	62	102
4	66	90

* 5×10^4 irradiated cells were added to each Petri dish to form the feeder layers.

It is evident that the culture systems described here have the essential properties that will allow a highly versatile and precise experimental approach to many problems of animal cell physiology and biochemistry. They provide populations of individual cells that multiply in suspension, from which representative samples may be taken at will. During the logarithmic phase the number of cells that will form colonies on a glass surface without feeder cells is a constant and high proportion of the total population, and the amounts of RNA, DNA, and protein per cell are constant.[7] In these respects there is a marked similarity between these populations of animal cells and many bacterial populations.

Summary.—When animal cells of two different strains were withdrawn from populations growing in suspension, they multiplied, without an appreciable lag, to form colonies. While the percentage of cells that formed colonies varied widely when they were plated from the stationary phase of growth, 75 per cent of the cells gave to colonies when plated at any time during the logarithmic period. Almost 100 per cent of the cells in the logarithmic phase grew into colonies if they were plated over feeder layers of X-irradiated cells, showing that all, or nearly all, of the cells were capable of multiplying if provided with the proper nutritional environment. The cell-culture systems described should facilitate a variety of studies on the physiology and biochemistry of animal cells.

We wish to acknowledge our gratitude to Dr. R. C. Parker for a great deal of helpful discussion and advice during the course of this work.

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ON THE DISTRIBUTION OF PURINE AND PYRIMIDINE BASES IN THE NUCLEIC ACID OF TOBACCO MOSAIC VIRUS

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Reddi and Knight[1] have recently investigated the products of ribonuclease digestion of the nucleic acids of several strains of tobacco mosaic virus. After the enzyme had been allowed to act on the isolated ribonucleic acids, the trichloroacetic acid–precipitable fraction (hereafter called "core") of each digest was analyzed to determine the molar ratios of purine and pyrimidine bases. As might be expected from the specificity of pancreatic ribonuclease,[2] the core material was found to contain several times as many purines as pyrimidines, whereas the ratio of purines to pyrimidines is close to unity in the whole nucleic acids. And just as no significant differences have been found in the over-all composition of the nucleic acids of the strains of tobacco mosaic virus,[3] no significant differences were observed in the base compositions of the cores.

In the present study an attempt is made to determine from theoretical considerations whether the similar compositions of the cores are indicative of some common arrangement of purines and pyrimidines in the nucleic acids. In order to do this, a hypothetical nucleic acid is considered to undergo the treatments described by Reddi and Knight and to serve as a control in their experiments. The control material is assumed to be a single long-chain nucleotide polymer with the characteristic ribose-to-phosphate linkages of a ribonucleic acid and to have the same relative numbers of adenylic, cytidylic, guanylic, and uridylic acid residues as do the nucleic acids of the viral strains. However, the sequence of these residues along the hypothetical polymer is assumed to be completely random, and for this reason it serves as a control; if the core compositions for the viral-strain nucleic acids do not differ from the core composition which would be obtained for the random polymer, then they are not necessarily indicative of any specific nucleotide sequence common to the strains. The validity of this test can be appreciated if it is remembered that the random arrangement is not any particular sequence of nucleotide residues but is a mathematical composite resembling a majority of the vast number of possible sequences. If the nucleic acids of the viral strains have core compositions consistent with such a model, it can be concluded that they are typical by this criterion, but not that the strains are identical or even similar with respect to their particular nucleotide sequences.

The question of how closely a viral nucleic acid resembles a random polymer has another, more general significance. Recent experiments[4] have indicated that a

molecule of viral ribonucleic acid having as few as 1,000 nucleotide residues can by itself bring about mosaic infection in plants and the production of complete nucleoprotein virus particles. Thus whatever additional information the cell may require in order to begin synthesizing the abnormal or viral materials must be somehow coded into a sequence of no more than 1,000 nucleotides. The number of different possible sequences of this length is of the order of 10^{602}, a number which dwarfs any conceivable figure for the number of different kinds of molecules actually involved in life-processes. But if there is any simple regularity in the nucleotide sequence, such as a repetition of one pattern involving only a few residues, then the possibility for information storage is severely limited. One indication of the presence of such a simple repeat in viral nucleic acid would be a core composition which differed significantly from that of a random sequence.

The Random Nucleic Acid.—Consider a single, unbranched polynucleotide chain of A purines and B pyrimidines in a random sequence. The chain may be conveniently thought of as a series of inter-pyrimidine segments containing 0, 1, or more purines, with each such segment separated from its neighbors (or "neighbor," in case of an end segment) by single pyrimidines. If all A purines are arranged in the $B + 1$ segments in a random manner, the probability that a given purine will lie in any one of the segments is the same for all segments and is therefore equal to $1/(B + 1)$. If A and B are both large, the variable n_N, representing the number of segments having just N purines, will be distributed according to the Poisson law:

$$n_N = \frac{Be^{-R_0}R_0^N}{N!}, \qquad \text{where } R_0 = \frac{A}{B}. \tag{1}$$

Ribonuclease Digestion and Precipitation of the Core.—Since pancreatic ribonuclease appears to cleave the nucleic acid chain only at one side of each pyrimidine residue (hydrolyzing the phosphoester bond at the ribose 5′ position), the fragments after digestion will consist of the original purine segments (note that some are of zero length), each attached to a terminal pyrimidine.[2]

At this point it is necessary to make some assumption as to the selective action of 6 per cent trichloroacetic acid (as used by Reddi and Knight) in precipitating the various fragments in the digest. For algebraic simplicity and for want of any empirical information on the solubilities of polynucleotides under these conditions, the following will be assumed: (1) that no fragments containing less than some critical number M of nucleotide residues (i.e., $M - 1$ purines) are precipitated; (2) that all fragments containing more than M residues are precipitated; and (3) that some fraction $0 \leq f < 1$ of the fragments containing just M residues are precipitated. According to these conditions, the fraction of the complete digest (in terms of nucleotide residues) which is precipitable is

$$F = \frac{fn_{M-1}M + \sum_{N=M}^{\infty} n_N(N+1)}{A + B}. \tag{2}$$

The molar ratio of purines to pyrimidines in the precipitate is

$$R = \frac{fn_{M-1}(M-1) + \sum_{N=M}^{\infty} n_N N}{fn_{M-1} + \sum_{N=M}^{\infty} n_N}. \tag{3}$$

The expressions for F and R in the case of a random nucleic acid are obtained by substituting n_N from equation (1) into equations (2) and (3) and simplifying:

$$F = \frac{e^{-R_0}}{R_0 + 1}\left[\frac{fMR_0^{M-1}}{(M-1)!} + \sum_{N=M}^{\infty} \frac{R_0^N(N+1)}{N!}\right], \tag{4}$$

$$R = \frac{fR_0^{M-1}(M-1) + (M-1)! \sum_{N=M}^{\infty} \frac{R_0^N}{(N-1)!}}{fR_0^{M-1} + (M-1)! \sum_{N=M}^{\infty} \frac{R_0^N}{N!}}, \tag{5}$$

where R_0, the ratio of purines to pyrimidines in the undigested polymer, is equal to 1.19 for tobacco mosaic virus nucleic acid.

Although F and R appear to depend on the two parameters f and M, they can be uniquely determined by a single parameter $P = M + f$. This is true because, for any such number P, there is only one combination of an integer (i.e., M) and a fraction (i.e., f) which when added together will yield that number. Therefore, for each assumed value of P, the corresponding values of F and R can be computed and by repeating the calculations for several P values, a plot can be obtained in which R is given as a unique function of F. Thus, although the exact nature of the selective precipitation is not known, it is possible to predict for the random nucleic

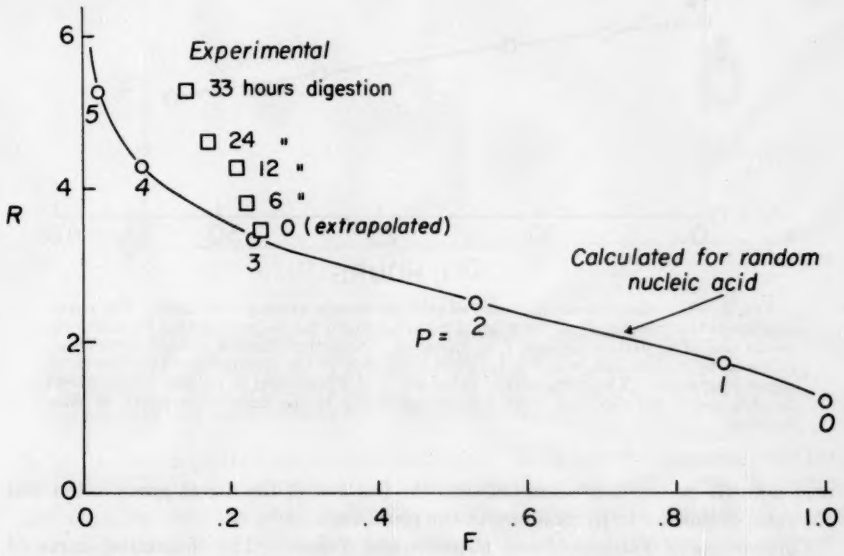

FIG. 1.—A comparison of the ribonuclease digest of tobacco mosaic virus nucleic acid ("experimental") with that of a randomly arranged ribonucleotide polymer of the same over-all base composition. The curve indicates the fraction, F, of the latter digest which would be precipitated by trichloroacetic acid and the ratio, R, of purines to pyrimidines in the precipitate for various values of the precipitation parameter, P. The tendency of the experimental points to fall farther from the curve with increasing digestion time probably reflects a "spontaneous" cleavage of nucleic acid at sites other than those known to be attacked by ribonuclease.

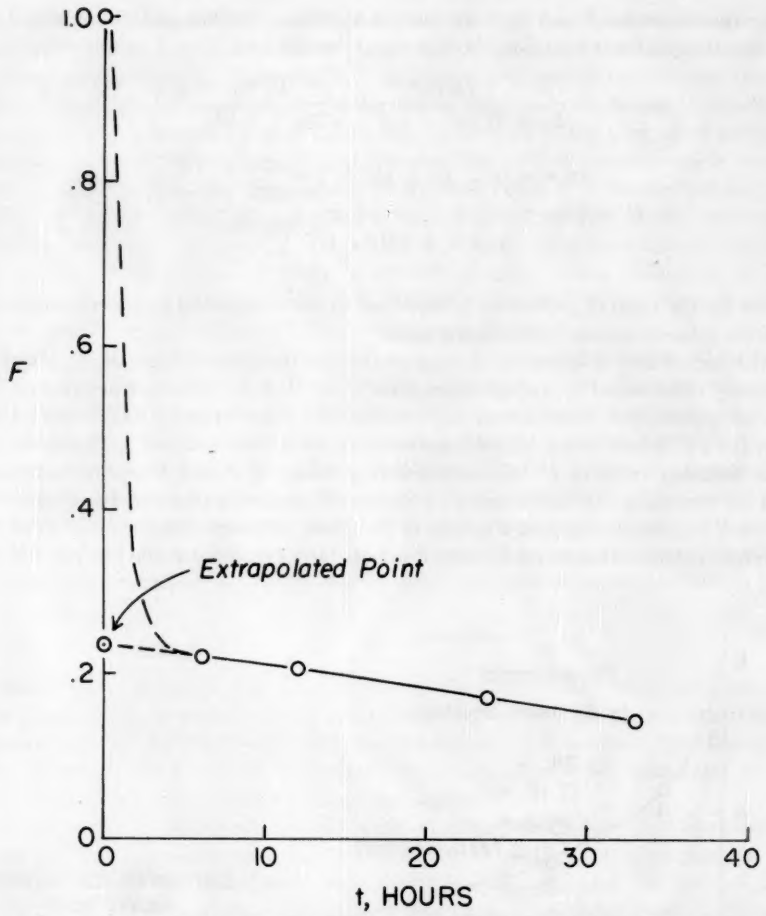

FIG. 2.—The ribonuclease digestion of tobacco mosaic virus nucleic acid. The curve represents the fraction, F, of the original material which can be precipitated by trichloroacetic acid after various periods, t, of digestion. Note that there is a rapid initial loss, followed by a slow loss, which is believed to be due to the spontaneous breakdown of digest fragments. The extrapolated value at $t = 0$ is presumed to be that which would be obtained if the digestion went entirely according to the known specificity of ribonuclease.

acid with $R_0 = 1.19$ a relation between the fraction of the digest precipitated and the ratio of purines to pyrimidines in the precipitate.

Comparison of Theoretical and Experimental Results.—The theoretical curve of R plotted against F for the random nucleic acid is compared in Figure 1 with the experimental points determined by Reddi and Knight for tobacco mosaic virus nucleic acid. Some rather large discrepancies will be noted. These may be attributed to one or more of the following: (1) a distribution of purines between successive pyrimidines which does not follow the Poisson law; (2) a breakdown of

nucleic acid in some manner other than according to the known specific action of pancreatic ribonuclease; (3) a manner of selective precipitation greatly different from that which has been assumed here. Evidence will now be given which indicates that the discrepancies between theory and experiment may be due primarily to the second of the possible causes listed.

The "Spontaneous" Breakdown of Nucleic Acid.—In Figure 2 the fraction of the viral nucleic acid digest precipitable by trichloroacetic acid is plotted as a function of digestion time. It will be seen that there was at first a rapid loss of precipitable material which was essentially completed within 6 hours. This was followed by a slower loss, which continued for the full period of observation without any apparent deceleration. Analogous trends are seen in Figure 3, where the ratio of purines

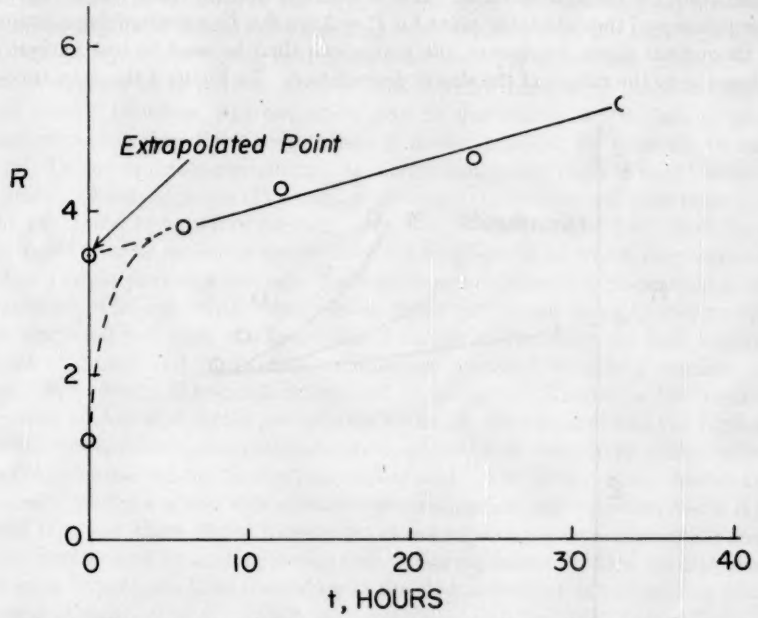

FIG. 3.—The ribonuclease digestion of tobacco mosaic virus nucleic acid. The curve represents the ratio, R, of purines to pyrimidines in the material precipitable by trichloroacetic acid after various periods, t, of digestion. As in Fig. 2, there is a rapid initial change, followed by a slow change. The extrapolated value at $t = 0$ is again presumed to be that which would be obtained in the absence of the spontaneous breakdown.

to pyrimidines is plotted against digestion time. Both curves suggest strongly that more than one mechanism was acting during the digestion period. The investigators reported also that when the nucleic acid was incubated in the absence of any known enzyme, there was a slow but appreciable loss of trichloroacetic acid–precipitable material. (The "spontaneous" breakdown of similar preparations has also been noted by Cohen and Stanley,[5] Northrup and Sinsheimer,[6] and others.) These results would suggest that in Figure 2 only the rapid, initial loss of precipitable material is due to the specific action of the ribonuclease and that the slower loss must be attributed to some other manner of breakdown. With this assumption,

the curves in Figures 2 and 3 have been extrapolated to zero digestion time in such a way that the trend due to the slow breakdown is continued and the trend due to the ribonuclease digestion is ignored. The extrapolated values are then presumed to be the values of F and R which would be obtained for the core material if the breakdown were due entirely to the known specific action of ribonuclease. In Figure 1 it will be seen that the point corresponding to these values lies very close to the theoretical curve for the random nucleic acid. The extrapolated point almost coincides with the calculated point for $P = 3$, which would indicate that all the digest fragments consisting of 4 or more nucleotide residues, and none smaller, are precipitated as core.

As the digestion period is increased, the experimental points fall farther and farther from the theoretical curve. If it is assumed that the discrepancies between these points and the calculated point for $P = 3$ are due to a gradual degradation of the theoretical digest fragments, the points can then be used to test various hypotheses as to the nature of the slower degradation. In Figure 4 the data are com-

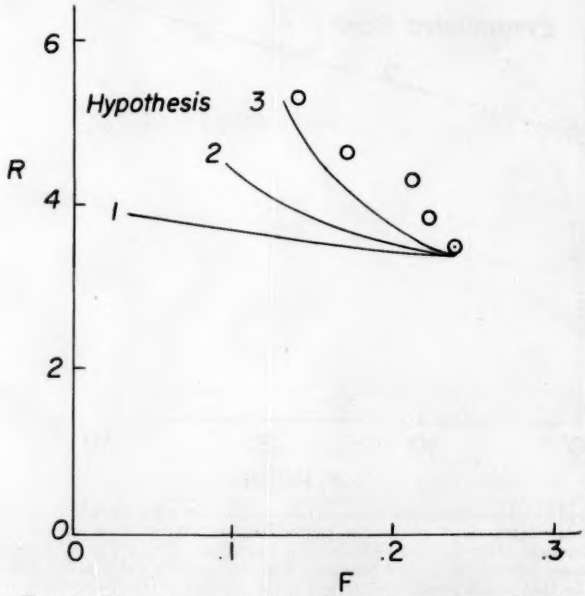

FIG. 4.—Plot similar to Fig. 1. Here the experimental points from Fig. 1 are compared with curves calculated for three different hypotheses as to the manner in which the fragments after ribonuclease digestion break down spontaneously. Hypothesis 3, which comes closest to fitting the data, postulates that by some unknown mechanism only the terminal pyrimidines are removed from the digest fragments.

pared with the curves calculated from three such hypotheses, which are as follows: (1) random breakage of phosphoester bonds—that is, within any specified interval of time the probability of a given bond being broken is the same for all such bonds; (2) random breakage (as defined above) of only those bonds connecting the ter-

minal residues to the rest of the chain; and (3) random breakage of only those bonds connecting the terminal pyrimidines to the rest of the chain. While none of these curves are in good agreement with the point, it will be noted that hypothesis 3 comes closest to fitting the data. This observation suggests that there is some specificity involved in the slower breakdown, though no chemical explanation for removal of terminal pyrimidines from the digest fragments can be offered at this time.

Conclusions.—The experimental results for the digestion of tobacco mosaic virus nucleic acid appear to be consistent with those which would be obtained with a randomly arranged ribonucleotide polymer of the same over-all base composition as the viral nucleic acid. This finding does not imply the lack of a definite sequential arrangement of residues in the viral nucleic acid. It does suggest, however, that the arrangement consists of something more complex than the mere repetition of one simple pattern involving only a few residues. For this reason, ribonuclease digestion appears to be quite limited as a method for comparing the structure of nucleic acids. However, this limitation may be due mostly to the lack of good methods for separating digest fragments. If it were possible, for example, to analyze only those fragments containing 7 or more residues, the method might become very useful. From equation (1) it can be calculated that a nucleic acid of molecular weight 10^6 would be expected to yield only 1 or 2 heptanucleotides. Such fragments would then be more characteristic of the molecules from which they were obtained and might provide a means of distinguishing or identifying ribonucleic acids.

Summary.—The pancreatic ribonuclease digest of tobacco mosaic virus nucleic acid is compared with that of a hypothetical nucleic acid in which the same relative numbers of purine and pyrimidine residues are arranged in a long random sequence. Specifically, the values determined by Reddi and Knight for the fraction of the viral nucleic acid digest precipitable by trichloroacetic acid and the ratio of purines to pyrimidines in the precipitate are compared with the corresponding values that would be expected for the random nucleic acid. The agreement is excellent if allowance is made for a slow side effect in the viral nucleic acid digestion and if it is assumed that just those digest fragments containing four or more nucleotide residues are precipitated by trichloroacetic acid. The experimental data are also used to test some hypotheses as to the nature of the slow side effect accompanying ribonuclease digestion.

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* United States Public Health Service Fellow of the National Cancer Institute.

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*MOLECULAR BASIS OF BIOLOGICAL STABILITY TO HIGH TEMPERATURES**

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One of the most plausible explanations for the ability of thermophilic organisms to grow at temperatures that usually are lethal to other organisms is that they possess relatively heat-stable proteins. Substantial support for this hypothesis recently has been furnished by Koffler and his associates,[1, 2] who showed that cytoplasmic proteins obtained from thermophilic bacteria are strikingly more heat-stable than those from mesophiles. Their experiments extend previous reports[3–7] that the activity of certain enzymes from thermophiles is more heat-stable than that of similar preparations from mesophilic cells. Unfortunately, the available data, except Campbell's,[6] were obtained with impure enzymes and thus do not enable one to distinguish between inherent thermostability and external stabilization. Campbell's experiments, however, were performed with purified enzymes. He showed that crystalline amylase isolated from cultures of *Bacillus coagulans* grown at 55° C. is markedly more heat-stable than a similar isolate from cultures grown at 35° C. Though one could argue that even crystalline proteins may contain stabilizing contaminants in minute yet effective concentrations, the most reasonable explanation for this finding is that the observed difference in relative thermostability is due to molecular differences and not to external substances. Campbell's observation confirms our own conclusions regarding the relative thermostability of flagella from thermophiles. These experiments were presented in preliminary accounts before[2, 8] and are described more fully in this paper.

Bacterial flagella are helical organelles made up of fibrous proteins[9–15] recently named "flagellins."[11] The characteristic appearance of flagella as observable in the electron microscope can be destroyed by acid,[9, 10, 15, 16] heat,[2, 8] urea,[2, 17] and acetamide.[2] Apparently flagella "dissociate" into their component units or multiples thereof; this seems true for the action of acid below pH 3–4[9, 14, 15] and is probably true also for the effect of the other agents mentioned. Suspensions of flagella are viscous, but their viscosity decreases markedly as the flagella dissociate. Such a decrease therefore can be used as a measure of stability. The advantage of using flagella as a test system is that highly purified preparations from many different organisms can be made rather easily by the same method involving fractional centrifugation (with or without fractional precipitation with ammonium sulfate). Moreover, because flagella are organized aggregates of protein molecules, they lend themselves to a study of intermolecular as well as intramolecular stability.

MATERIALS AND METHODS

Organisms.—The mesophilic bacteria used in this study were the following: *Proteus vulgaris* Purdue M, *Escherichia coli* ATCC 26, *Serratia marcescens* 8 UK,

Bacillus megaterium KM, and *B. subtilis* 712. The following thermophilic strains of the genus *Bacillus* were used: *B. stearothermophilus* NCA 2184, *B.* sp. Purdue CD, *B.* sp. Texas 11330, and *B.* sp. Nebraska 39. We are grateful to various colleagues in other laboratories for making some of these cultures available to us.

Isolation of Flagella.—The above organisms were usually grown in a medium consisting of the following (in gm/liter): gelysate, 10; trypticase, 5; yeast extract, 1; dextrose, 2.5; sodium chloride, 5; dibasic potassium phosphate, 2.5. The medium, in 125-ml. quantities, was placed in 500-ml. Erlenmeyer flasks, sterilized, and, after cooling to the appropriate growth temperature, inoculated with 5 ml. of a broth culture. The cultures were incubated until they had reached the end of the logarithmic growth phase or the early part of the maximum stationary phase (8–12 hours). Mesophiles were grown at 20° C., thermophiles at 60° C. Aeration was increased by agitation on reciprocating shaking machines.

The cells were harvested by centrifugation at 3,900 × *g* for 30 minutes (100-ml. bottles; 4,600 rpm; International Refrigerated Centrifuge, Model PR-1). Usually 6 liters of culture was handled in one experiment. The cells were resuspended in 400–600 ml. of 0.9 per cent saline solution and washed by centrifugation. After the cells had been washed, they were resuspended in 200–300 ml. of cold saline solution and placed in a 500-ml. ground-glass-stoppered bottle that contained *ca.* 100 gm. of frozen saline solution. The bottle was placed on a shaking machine ("Miracle Paint Rejuvenator") and shaken at a rate of 620 $1^1/_4$-inch strokes/min for 20 minutes. This step broke off most of the flagella from the cell bodies. The shaken suspension was then centrifuged twice at 3,900 × *g* for 45 minutes at 0°–4° C. The centrifugate was discarded each time. The supernatant liquid was then brought to 0.1 saturation with ammonium sulfate and allowed to stand for 8–12 hours. The precipitate was sedimented at 3,900 × *g* for 45 minutes and then discarded. The supernatant liquid was brought to 0.5 saturation with ammonium sulfate and allowed to stand for 8–12 hours. The precipitate was concentrated by centrifugation at 3,900 × *g* for 45 minutes and then centrifuged at 25,000 × *g* for 5 minutes (19,000 rpm; high-speed attachment of the International Refrigerated Centrifuge). The supernatant liquid was discarded. The surface of the pellet and the inside walls of the tube were washed with distilled water. By discarding the 0–0.1 and 0.5–1.0 fractions, one eliminates a large portion of nonflagellar materials.[15] The precipitate was then resuspended in 40 ml. of distilled water and centrifuged twice at 25,000 × *g* for 30 minutes; the centrifugate was discarded each time. The supernatant liquid was centrifuged twice at 59,000 × *g* for 35 minutes (30,000 rpm; No. 40 rotor, Spinco Model L Preparative Ultracentrifuge). The purified flagella were stored at 0°–4° C. until used. A second method of growing cells and isolating flagella was used for some of the experiments.[18] However, inasmuch as the procedural variations did not seem to influence the results, details regarding these variations are omitted.

Determination of Purity.—To determine dry weights, the samples were placed in tared glass or aluminum-foil dishes, dried at 110° C. for 4 hours, and weighed on a microchemical balance. Total nitrogen was determined according to the method of Willits and Ogg.[19] Carbohydrate (as hexose) was determined by the anthrone method of Loewus.[20] Nucleic acid was determined according to the method of Warburg and Christian;[21] a correction for nonspecific dispersion of radiant energy

was made as described by Weibull.[9] As mentioned previously, flagella dissociate below pH 3–4. Flagella thus treated are no longer centrifugable at 59,000 × *g* for 30 minutes. It was found that the sediment obtained under such conditions contains the bulk of nonflagellar impurities.[9, 15] The absence of such material is perhaps the most informative single criterion for the purity of isolates. In this study the routine procedure was exposure of the flagella to pH 2 at 26° C. for 30 minutes, followed by centrifugation at 59,000 × *g* for 30 minutes ("pH 2 insoluble material"). The pellet, if any, was transferred to a tared glass dish and weighed as described. Usually the preparations used were estimated to be approximately 95–99 per cent pure. The ranges of values encountered for preparations from the eight organisms employed in this study were as follows: total Kjeldahl nitrogen, 15.8–16.4 per cent; carbohydrate, 0.1–0.6 per cent; nucleic acid, 0.1–0.5 per cent; "pH 2 insoluble material," 0.2–0.7 per cent.

Determination of Viscosity.—To test the stability of flagella to heat, 2 ml. of a suspension was placed in a viscometer of the Ostwald type. The initial viscosity was determined at 20° ± 0.05° C. after a period of equilibration of 1 minute. The instrument was then immersed in a water bath at temperatures varying from 40° to 80° C. Measurement of exposure time was begun after an equilibration period of 1 minute. After an interval of time, the viscometer was removed, quickly cooled to 20° C., and the flow time of the suspension was redetermined. The heating then was continued in increments of 5 or 10 minutes until the total exposure time to a given temperature was 30 minutes. To test the stability of flagella to various chemical agents, 1 ml. of suspension was placed in a viscometer, and the instrument was placed in a water bath at 20° C. One milliliter of the solution (at 20° C.) containing twice the desired final concentration of the compound under study was added to the suspension of flagella. The flow time of the mixture was determined at intervals over the course of an hour or longer.

The results were expressed in terms of relative viscosity, $\eta_{rel} = \eta/\eta_0$; specific viscosity, $\eta_{sp} = \eta_{rel} - 1$; or reduced viscosity, $\eta_{rel} = 1\ \eta_{sp}/C$, where η is the viscosity of the solvent plus flagella, η_0 is the viscosity of the solvent, and C is the concentration of flagella in mg/ml. The ratio η/η_0 was assumed to be equal to the ratio of the flow time of the flagellar suspension to that of the solvent. The relative viscosity of flagellar suspensions in the concentrations employed was not significantly affected by the velocity gradient, changes in pH from 4.4 to 9.1, and small concentrations of sodium chloride (0–0.15 *M*).

RESULTS

Relative Thermostability of Thermophile Flagella.—The effect of heat on the stability of flagella (usually 0.5 mg/ml) was determined. Decrease in the viscosity of flagellar suspensions at various temperatures was used as a measure of thermal destruction.[8] The products of this "disintegration" (dissociation) are nondialyzable and are precipitable by ammonium sulfate, but they cannot be sedimented at the RCF sufficient to remove whole flagella. Figure 1 shows the effect of heat on the flagella from *E. coli*, a mesophile. Figure 2 illustrates the stability of flagellar from *B.* sp. 11330, a thermophile. From experiments similar to these, the data summarized in Table 1 were obtained. Table 1 shows the decrease in the specific viscosity of flagellar suspensions after exposure to various tempera-

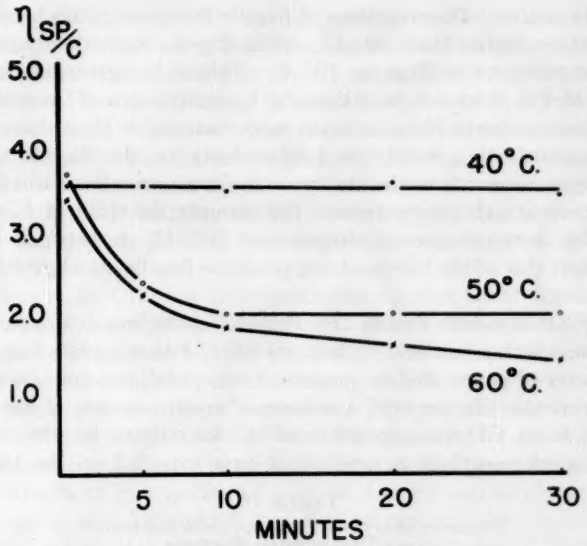

FIG. 1.—Effect of various temperatures on the reduced viscosity of suspensions of flagella from the mesophile *Escherichia coli*.

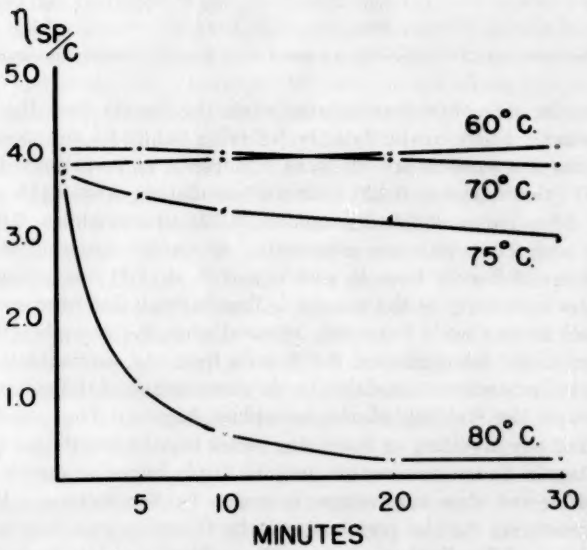

FIG. 2.—Effect of various temperatures on the reduced viscosity of suspensions of flagella from the thermophile *Bacillus* sp. 11330.

tures for 20 minutes. Disintegration of flagella from mesophiles becomes marked at temperatures higher than 50° C., while flagella from thermophiles remain intact at temperatures as high as 70° C. This is in agreement with the experience of McCoy,[22] who showed that the H-agglutinogen of *Clostridium thermosaccharolyticum*, a thermophile, is much more heat-stable than that of *C. butyricum*, a mesophile. In general, the heat stability of the flagella used in the present study corresponds to the ability of the organisms from which they were isolated to grow at high temperatures. For example, the strain of *B. subtilis* used, for which the maximum growth temperature (52° C.) is between that of the mesophiles and that of the thermophiles, produces flagella which are intermediate in heat stability.

Inability To Demonstrate Factors That Protect Flagella from Destruction by Heat.—The question whether the relative heat stability of thermophile flagella is an inherent property or is provided by protective materials[23] was considered in a number of experiments. In the first, a mixture of equal amounts of flagella from *P. vulgaris* and *B.* sp. CD was exposed to 60° C. for varying lengths of time. The mixture behaved essentially as one would have expected on the basis that the

TABLE 1*

THERMOSTABILITY OF FLAGELLA FROM MESOPHILIC AND THERMOPHILIC BACTERIA

FLAGELLA FROM	PER CENT INITIAL SPECIFIC VISCOSITY AFTER 20 MIN. AT °C.						
	40	50	60	65	70	75	80
P. vulgaris	99	67	18	...	..	..	..
E. coli	100	56	36	...	..	..	..
B. megaterium	100	72	28	...	..	..	..
B. subtilis	...	98	79	48	31	..	..
B. stearothermophilus 2184	...	..	...	100	..	43	18
B. sp. CD	...	..	...	100	99	94	41
B. sp. 11330	100	..	105	...	96	79	5
B. sp. 39	100	..	112	...	94	56	14

* Samples were taken every 5–10 minutes over a period of 30 minutes. Viscosity measurements were made at 20° C.

flagella from the mesophile disintegrated while the flagella from the thermophile remained intact. From kinetic data the following values for the specific viscosity of the flagellar suspensions are given as examples: at zero time—0.059 (mesophile), 0.061 (thermophile), 0.120 (mixture calculated), and 0.115 (mixture observed); at 30 minutes—0.010 (mesophile), 0.059 (thermophile), 0.069 (mixture calculated), and 0.061 (mixture observed). A similar situation was observed when a mixture of flagella from *B. subtilis* and *B.* sp. CD was exposed to 65° C. If the relative instability of the mesophile flagella were due to external labilizing factors,[23] such factors could have been released when the mesophile flagella disintegrated and might have affected the flagella from the thermophile. Similarly, the presence of protective materials[23] in the thermophile, if diffusible, might have left its mark on the stability of the mesophile flagella. The possibility exists, of course, that any labilizing or stabilizing factor may be specific for a given preparation of flagella under examination, may be firmly bound or unable to reach the necessary sites, and thus may escape detection by this method. However, the simplest explanation for the previous and the following experiments is that the relative stability of flagella depends upon the molecular architecture of the flagella themselves rather than on extraneous materials.

Cell-free extracts of *B.* sp. CD were prepared by oscillation of a suspension of this organism in distilled water for 90 minutes in a Raytheon 9-kc. Magnetostriction Oscillator. The debris was centrifuged at 25,000 × *g* for 1 hour. The clear supernatant liquid, containing 40 per cent of the total dry weight of the cells, was concentrated to dryness under vacuum at 23° C. and stored at 0°–4° C. until needed. The cell-free extract and flagella from *B. subtilis* in concentrations of 0.5 mg/ml were mixed, and the stability of the flagella under these conditions was compared to their stability in the absence of thermophile material. The cytoplasmic constituents isolated from the thermophile exerted no appreciable protective effect.

Flagella from *B.* sp. CD were disintegrated by exposure to 85° C. for 15 minutes, to pH 2 for 30 minutes at 26° C., or to sonic oscillation (Brush Development Company Hypersonic-Transducer, Model Bu-101-A, 15 minutes). It was hoped that a protective factor contained in the thermophile flagella would be released but not destroyed by one or more of these treatments. The preparations disintegrated by heat or acid (after neutralization) were mixed in equal amounts (usually 0.5 mg/ml) with flagella from *B. subtilis*. The sonically disintegrated thermophile flagella were mixed with flagella of *P. vulgaris* (0.5, 1.0, 1.5, and 2.0 mg/ml of extract with 1.0 mg/ml of flagella; also, 3.0 and 6.0 mg/ml of extract with 3.0 mg/ml of flagella). No significant stabilization of the flagella from *B. subtilis* and *P. vulgaris* was observed at 65° and 55° C., respectively. Also, the effect of calcium ions, previously implicated in the stabilization of proteins,[24–26] was tested (equal volumes of 0.05, 0.1, 0.5, and 1.0 *M* solutions of calcium chloride and suspensions of flagella from *B. subtilis*, 0.5 mg/ml; 65° C.). No stabilizing effect could be demonstrated.[27]

The possibility of adsorbing a hypothetical protective factor was tested by determining whether thermophile flagella treated with various adsorbing agents would become less heat-stable.[27] Two milliliters of a suspension of flagella from *B.* sp. CD (3.0 mg/ml) was incubated for 15 minutes at 26° C. in the presence of Dowex 50 (H form; 20 and 40 mg/ml), Dowex 2 (OH form; 20 and 40 mg/ml), or glass beads (3 mm, 5 beads/ml); the suspensions were shaken gently. The adsorbing agents were removed by centrifugation, and the flagella in the supernatant liquid were examined for heat stability at 60° C. Even after treatment with exchange resins or beads, the flagella remained unaffected by that temperature.

Enzymatic removal of a possible protective material was attempted by exposure of flagella from *B.* sp. CD to the action of trypsin, ribonuclease (RNAse), and desoxyribonuclease (DNAse). Trypsin was assayed by a method described by Crewther,[28] and RNAse and DNAse by the method of Roth.[29] To eliminate the possibility that the flagella might be inhibitory, the activity of these enzymes on standard substrates (gelatin, yeast RNA, and sperm DNA) was determined in the presence and in the absence of flagella. The presence of the flagella had no effect on these enzymes. Sufficient enzyme was added to flagellar preparations to hydrolyze a weight of substrate that was from 20 to 40 times greater than the total amount of flagella present. After incubation with trypsin, RNAse, and DNAse at various temperatures and for varying lengths of time, the thermophile flagella were just as thermostable at 65° C. for 20 minutes as they had been before enzymatic treatment.

The Unlikelihood of Disulfide Linkages Being Responsible for the Relative Stability

of Thermophile Flagella.—The possibility that thermophile flagella are more stable because of more effective disulfide bonding did not seem likely, because of the fact that the cysteine-cystine contents of mesophile and thermophile flagella are low and are similar regardless of the source (0.54–0.75 per cent).[14] Moreover, the rapid dissociation of flagella below pH 3–4 speaks against the existence of intermolecular disulfide bridges. Nevertheless, the effect of sodium thioglycolate, an agent known to break —S—S— bridges, on the stability of flagella was determined. Suspensions of flagella from *B. stearothermophilus* 2184 ($\eta_{rel.}$ 1.23) and *B.* sp. CD ($\eta_{rel.}$ 1.30) were mixed with thioglycolate in the presence of urea (final concentrations: 0.2 *M* thioglycolate and 5 *M* urea in *M*/60 phosphate buffer, pH 8). Under the same conditions a 5 per cent solution of ovalbumin was appreciably denatured (after 24 hours at 26° C. the solution was diluted fivefold and the pH adjusted to 4.8; over 25 per cent of the ovalbumin was precipitated, while only 5 per cent of it precipitated when thioglycolate was absent). Flagellar suspensions were incubated for varying lengths of time, and their stabilities were determined by viscosity measurements. Thioglycolate did not affect the viscosity of these suspensions, even after contact for 110 hours.

Evidence for More "Effective" Hydrogen Bonding in Thermophile Flagella.—If the relative thermostability of flagella from thermophilic bacteria is due to hydrogen bonding,[23, 30] they should be more resistant than mesophile flagella to compounds regarded as hydrogen-bond breakers, such as urea and acetamide. The effect of urea on the viscosity of a suspension of flagella from *B. subtilis* is shown in Figure 3. To disintegrate thermophile flagella to the same extent, one needs to increase the concentration of urea to 9 *M;* at a level of 6 *M*, urea does not exert any observable effects on thermophile flagella. This is shown in Table 2, in which the relative stabilities of four mesophile and four thermophile preparations are compared. Disintegration by urea, like that by heat, is accompanied by the loss of the characteristic flagellar structure; similarly, the products are nondialyzable and cannot be sedimented at the RCF at which whole flagella can be centrifuged. The data indicate that the thermophile flagella are not only more heat-stable than are the mesophile flagella but also more resistant to the action of urea. They are also more resistant to 10 *M* acetamide, as is shown in Table 2. The experiments with acetamide were performed in a manner similar to those previously discussed, but for the sake of brevity only end-point data are presented.

Inability To Demonstrate Factors That Protect Flagella from Disintegration by Urea or Labilize Them to Its Action.—Evidence for the existence of possible stabilizing factors in the thermophile flagella from *B. stearothermophilus* 2184 or labilizing factors in the mesophile flagella from *P. vulgaris* was sought in experiments similar in design to those previously performed.

Cell-free extracts were prepared as before, except that the cells were exposed to oscillation for 30 instead of 90 minutes and the debris was removed by three consecutive centrifugations of 60 minutes each. The ash of whole cells was obtained by the method of Bates *et al.*[31] The addition of cytoplasmic constituents from the thermophile (2.5 mg/ml) to the mesophile flagella (2.0 mg/ml) or from the mesophile (2.4 mg/ml) to the thermophile flagella (1.2 mg/ml) did not markedly alter the stability of the flagellar preparations to 5 *M* urea. No stabilization or labilization was brought about when thermophile ash was added to mesophile

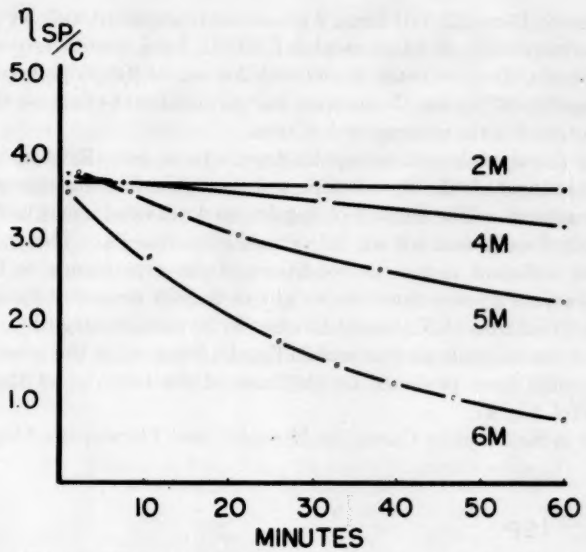

FIG. 3.—Effect of various concentrations of urea on the reduced viscosity of suspensions of flagella from the mesophile *Bacillus subtilis*.

TABLE 2*

STABILITY OF FLAGELLA TO UREA, ACETAMIDE, AND SODIUM DODECYLSULFATE (SDS)

	PER CENT INITIAL REDUCED VISCOSITY IN PRESENCE OF					
	6 *M* Urea				10 *M* Acetamide	0.0035 *M* Dodecylsulfate
	After Minutes					
FLAGELLA FROM	5	10	30	60	30	60
P. vulgaris	4	9	1	1	11	67
E. coli	16	9	7	7	...	...
B. megaterium	23	17	8	7	17	60
B. subtilis	83	72	43	21	13	63
B. stearothermophilus 2184	100	100	100	100	136	103
B. sp. CD	104	108	111	114	110	100
B. sp. 11330	100	100	100	100	94	100
B. sp. 39	103	103	104	107	...	...

* Samples were taken every 5 minutes over a period of 60 minutes. The data presented above were abstracted from experiments similar in execution to those shown in Fig. 3.

flagella or when mesophile ash was added to thermophile flagella (all in concentrations of 2.0 mg/ml).

The products of flagella disintegrated by heat (thermophile flagella at 80° C. for 30 minutes; mesophile flagella at 60° C. for 30 minutes), acid (prepared as before), and oscillation (20 minutes' treatment in a 9-kc. Raytheon Magnetostriction Oscillator) did not significantly change the behavior of whole flagella obtained from cells of the opposite stability type. The following concentrations were used in these experiments (mg/ml): whole and disintegrated flagella from *B. stearothermophilus* 2184, 1.2; from *P. vulgaris*, 2.0.

Treatment of flagella with Dowex 50 (H form; 2 gm. moist resin mixed with 5.0 mg. of thermophile flagella [in 4 ml] or 2.4 mg mesophile flagella [in 3 ml]; 26°

C. for 5 minutes), Dowex 2 (OH form, 2 gm. moist resin mixed with 5.0 mg. flagella [in 4 ml., thermophile; in 5 ml., mesophile]; 26° C. for 5 minutes), or small beads (5.0 mg. of Scotch-lite glass beads mixed with 5.0 mg. of thermophile or 5.0 mg. of mesophile flagella; 26° C. for 15 minutes) had no significant effect on the behavior of the flagella tested in the presence of 5 *M* urea.

Exposure of thermophile and mesophile flagella to trypsin, RNAse, and DNAse left the manner in which the thermophile and mesophile flagella responded to urea essentially unaltered. The amount of flagella used per experiment was 4.0 mg/ml of thermophile flagella and 3.0 mg/ml of mesophile flagella. The enzymes were used in levels sufficient under the conditions of the experiments to hydrolyze a weight of substrate greater than the weight of flagella present. Incubation was at 20° C. for 10 minutes (RNAse and DNAse) or 20 minutes (trypsin).

Mixtures of thermophile and mesophile flagella behaved in the presence of 5 *M* urea as one could have predicted on the basis of the behavior of the individual preparations (cf. Fig. 4).

Differences in the Titration Curves for Mesophile and Thermophile Flagella.—Wei-

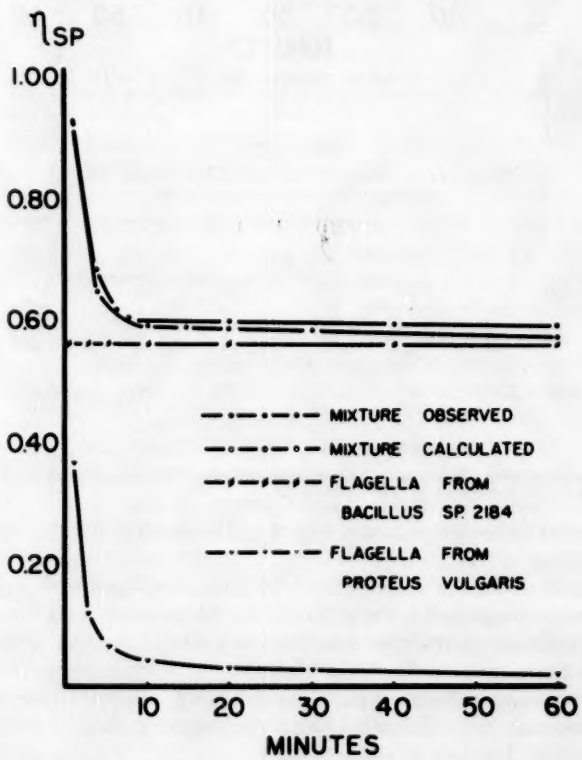

FIG. 4.—Effect of 5 *M* urea on the specific viscosity of mixed suspensions of flagella from the mesophile *Proteus vulgaris* and the thermophile *Bacillus stearothermophilus* 2184.

bull examined the titrimetric behavior of flagella from *P. vulgaris* and found that after disintegration of flagella at pH 2.5 the solution of flagellin acted as if additional titratable groups had become available.[32] In the intact flagellum such groups may not be titratable, because they are sterically inaccessible or combined in linkages. However, the difference in titration curves may also be due to changes in the pKa values of the ionizable groups after the flagellum has broken apart. In any case, it became of interest to determine whether the relative stability of thermophile flagella might be due to interactions in which these groups are involved. Titration curves were prepared according to the method described by Weibull,[32] and those for the mesophile flagella were similar to that described for *P. vulgaris*. The apparent increase in the number of titratable groups after dissociation of the flagella by acid cannot be correlated with the relative thermostability of the flagella. On the other hand, there is a striking difference in the total number of H^+ bound at pH 2.5 and 10.5. This is shown in Table 3.[33]

TABLE 3*

NUMBER OF TITRATABLE GROUPS IN FLAGELLIN FROM MESOPHILIC AND THERMOPHILIC BACTERIA

FLAGELLIN FROM	MIN. MOL. WT.	H^+ BOUND/10^5 GM AT pH		H^+ BOUND/MOLE AT pH	
		2.5	10.5	2.5	10.5
P. vulgaris	19,600	+120	−63	+24	−12
S. marcescens	20,800	+111	−53	+23	−11
B. subtilis	18,200	+102	−48	+19	− 9
B. stearothermophilus 2184	13,900	+ 58	−40	+ 8	− 8
B. sp. CD	15,200	+ 90	−32	+ 7	− 5
B. sp. 11330	16,700	+ 67	−28	+10	− 4

* The values presented in the table were taken from complete titration curves prepared as done by Weibull (*Acta Chem. Scand.*, **4** 260, 1950). The minimal molecular weights were calculated on the assumption that each flagellin molecule contains one N-terminal alanine (see Koffler *et al.*, *Arch. Biochem. and Biophys.*, **64** 509, 1956).

These data can be interpreted in at least two ways. (1) Mesophile flagella possess approximately twice as many acidic and basic groups as do thermophile flagella. This means that the flagellin molecules constituting mesophile flagella potentially have a greater opportunity for electrostatic attraction and repulsion than do those of which thermophile flagella are composed. Greater electrostatic attraction is not likely in the light of our other observations, but it is possible that the net stability of thermophile flagella is the consequence not only of more effective bonding between flagellin molecules but also of lesser repulsion between them. (2) Thermophile flagella possess the same number of titratable groups as do mesophile flagella, but these groups are more strongly acidic and basic in the former. For example, the carboxylate anions in thermophile flagella may be stabilized in such a way (probably through hydrogen bonds) as to lower the effective pKa of these carboxyl groups (interaction of the ammonium cation might increase the pKa of the amino groups).[34] Thus the total number of H^+ bound at pH 2.5 may be considerably less than the maximum acid binding capacity. Comparison of the maximum number of titratable groups at pH 1 or pH 1.5 therefore may reveal that both mesophile and thermophile flagella possess the same number.

The differences between the titration curves of mesophile and thermophile flagella need further study before one can distinguish definitely between the above possibilities. In any case, though, it is clear that the amphoteric properties of

mesophile and thermophile flagella differ remarkably and that these differences probably constitute useful clues regarding their architecture.

The Relative Stability of Thermophile Flagella to Sodium Dodecylsulfate.—Thermophile flagella apparently are more resistant to the action of sodium dodecylsulfate (SDS) than are mesophile flagella. For example, 3.5×10^{-3} *M* of SDS in *M*/60 phosphate buffer (pH 8) caused the partial disintegration of mesophile flagella but had no effect on thermophile flagella (cf. Table 2); to bring about the same degree of dissociation of thermophile flagella, the concentration of SDS had to be raised to 8.7×10^{-3} *M*.

The mechanism by which anionic detergents denature proteins is not completely understood, but it is thought that they first react with basic groups.[23] The greater resistance of thermophile flagella to the action of SDS, therefore, may be merely an expression of the fact that they possess fewer accessible basic groups than do mesophile flagella. On the other hand, in the above experiment the observed dissociation of the flagella did not occur until the molar concentration of SDS was greater than the molar concentration of titratable basic groups (for instance, the flagella from *B. subtilis* used were 6.25×10^{-4} *M* with respect to titratable basic groups, and the flagella from *B. stearothermophilus* were 5.2×10^{-4} *M*). Inasmuch as the nonpolar moiety of SDS might be expected to have an affinity for the nonpolar residues of the flagella,[30] it is possible that the dissociation of the flagella is caused by a disruption of hydrophobic bonds. This may be true especially for the thermophile flagella, the observed dissociation of which was brought about by concentrations of SDS considerably in excess of those that are likely to be bound electrostatically. The greater stability of thermophile flagella to SDS therefore may be a result of more "effective" hydrophobic bonding, but this interpretation needs to be examined further.

DISCUSSION

The remarkable ability of thermophile organisms to grow at high temperatures may be conferred by one or both of the following features: (1) the relative heat stability of their cellular components, a stability that may be either inherent or provided by protective materials, and (2) the rapid resynthesis of essential molecules damaged by heat.[4] Various types of substances likely to be present in the cytoplasm of organisms have been found to protect proteins from disorganization by heat,[23] among them calcium and other metallic ions,[24–26] sodium desoxyribonucleate,[35] certain proteins,[36] and cellular particles.[37] Certain impure, relatively heat-stable enzyme preparations have been shown to lose their thermostability on purification.[38] These findings make it imperative that studies regarding the nature of biological thermostability include a diligent search for protective materials.

Koffler and Gale showed that the striking relative heat stability of cytoplasmic proteins from thermophiles was not significantly diminished when "small particles" and nucleic acids were removed.[1] However, one could contend that a small amount of these materials remained, sufficient for protection. Perhaps more convincing were their experiments, in which cytoplasmic proteins from mesophiles and thermophiles were mixed and the heat stability of the mixture could be accounted for by the stabilities of the individual preparations. Campbell[5] showed that an amylase isolated from a facultatively thermophilic bacterium grown at

55° C. remained relatively heat-stable even when purified to the point of crystallization; a similar preparation obtained from cultures grown at 35° C. was not heat-stable. This work constitutes a valuable contribution on two accounts. First, the use of crystalline enzymes increases the likelihood that intrinsic stability rather than extrinsic stabilization was observed. Second, the use of the same organism for the production of relatively heat-stable and relatively heat-labile amylase decreases the possibility that the correlation between the stability of the enzyme and the growth temperature of the organism is fortuitous. His observations confirmed our earlier preliminary account,[8] more fully developed in this paper, regarding the heat stability of purified flagella.

We have shown that flagella from thermophiles are much more stable than flagella from mesophiles. Though it is impossible to state categorically that the observed relative stabilities are not brought about by protective or labilizing materials, the evidence strongly supports such a view. A persistent search for the existence of such substances has yielded no positive information.

The following facts speak against the importance of —S—S— linkages in holding flagellin molecules together in the flagellum. (1) Flagella contain only small amounts of cysteine-cystine, regardless of whether they have been produced by mesophiles or by thermophiles; each molecule of flagellin contains probably only one molecule of cysteine or cystine.[14] (2) Flagella dissociate under relatively mild conditions, for example, below pH 3–4.[9, 10, 14, 15, 16] (3) Thioglycolate in a concentration known to denature other proteins does not affect the stability of flagella; however, in the native flagellum, the —S—S— bridges may not be accessible.

Since thermophile flagella are more resistant than mesophile flagella to urea and acetamide, agents thought to break hydrogen bonds,[13, 30] it seems that more "effective" hydrogen bonding is involved in the structural stability of thermophile flagella. "Effectiveness" may depend on the number, strength, or location of the bonds (or on a combination of all three factors).

The observation that thermophile flagella might contain only half the number of titratable groups found in mesophile flagella suggests another explanation for the relative stability of the former. Flagellin molecules in the flagellum may face sites carrying charges of like sign. Because of the resulting electrostatic repulsion, such molecules would have a tendency to dissociate. The main difference between mesophile and thermophile flagella therefore may reside not in differences in bonding but in a greater or lesser tendency of the constituent flagellin molecules to repel each other.

Thermophile flagella are also more resistant than are mesophile flagella to sodium dodecylsulfate, an anionic detergent. This may be because thermophile flagella possess fewer basic groups to which the SDS can be anchored than do mesophile flagella. However, the amount of SDS necessary to cause the observed disintegration of flagella is in excess of that theoretically needed to neutralize the available basic groups, and the dissociation of flagellin molecules actually may come about through the breakage of hydrophobic bonds by the detergent. Hydrophobic bonding is thought to be due to the tendency of relatively nonpolar amino acid residues to avoid the aqueous phase and to adhere to each other.[30] Such bonding may play an important role in holding the flagellin molecules together in the flagellum. The nonpolar portion of SDS is more likely to stick to the nonpolar residues of the protein

than to enter the aqueous phase. This may result in the rupture of existing hydrophobic bonds. The relatively greater resistance of thermophile flagella to SDS therefore may be due to stronger, more numerous, or more strategically located hydrophobic bonds than occur in mesophile flagella.

In addition to the explanations mentioned, one has to consider the possibility that the stability of thermophile flagella is provided by novel types of bonding. No information regarding this has been discovered so far.

SUMMARY

Flagella from thermophilic bacteria are much more stable than similar structures from mesophilic bacteria. Since flagella are organized aggregates of fibrous proteins, this relative stability probably is a reflection of the forces holding these molecules together. The relative stability of flagella appears to be an intrinsic property of these structures, since no stabilizing or labilizing materials could be demonstrated. Thermophile flagella are also more stable than mesophile flagella in the presence of urea and acetamide, agents regarded as hydrogen-bond breakers, and of sodium dodecylsulfate, an anionic detergent. These findings are interpreted to mean that the relative stability of thermophile flagella is due to more numerous, stronger, or more strategically located hydrogen and/or hydrophobic bonds than are present in mesophile flagella. The latter seem to contain twice as many titratable groups as do the former. This suggests that under certain circumstances they might be under greater stress, created by the repulsion between charged groups having like signs.

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*ON EXCITATION OF BIOLOGICAL SUBSTANCES**

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It has been noted[1] that watery solutions of fluorescent dyes profoundly alter their optical behavior on freezing: their fluorescent emission disappears. On further lowering of the temperature, light emission reappears, but the emitted light differs from the earlier fluorescent light in having a longer wave length and coming from an excitation having a longer lifetime, answering, thus, to the formal definition of "phosphorescence." This change in properties is not limited to dyes. Certain proteins, amino acids, coenzymes, and steroid hormones, for instance, show analogous behavior.

With regard to its possible biological implications, this phenomenon deserves further study. Two questions suggest themselves: (1) Does the phosphorescent light originate from a triplet excitation or from a crystal phosphor? (2) Is this behavior a universal property of fluorescent substances? The experiments reported in this paper suggest answers to these questions.

There are several criteria which can be utilized in studies of luminescence to characterize an emission as arising from a metastable triplet level.

1. Triplet emission is shifted toward the longer wave lengths relative to the fluorescent emission.
2. Triplet emissions have a much longer lifetime than singlet emissions.

3. Triplet-state emissions obey first-order decay kinetics.
4. Triplet-state emissions are usually only slightly temperature-dependent, i.e., activation energy is negligible.
5. The molecule in the triplet state, having an unpaired electron spin, is paramagnetic.
6. Triplet emissions induced by polarized exciting light are slightly polarized or nonpolarized.
7. The molecule in the triplet state, being a chemical species in its own right with its own set of properties, will exhibit its own characteristic absorption spectrum.

For the work reported in this paper we have used Criteria 1–4 to aid in characterizing the electronic states involved.

METHODS

CRITERION 1: EMISSION SPECTRA

A. VISUAL

A knowledge of the fluorescent spectrum for a particular compound permits one to observe visually whether a subsequent delayed emission has undergone a wave-length shift. For example, tyrosine fluorescence resides exclusively in the ultraviolet, but when the compound is frozen in the presence of glucose, it exhibits, upon proper excitation, a pale blue afterglow. We recognize this shift of the emission toward longer wave lengths as presumptive evidence for the occurrence in the molecule of an electronic transition different from that responsible for the ultraviolet fluorescence. In this study most of our observations of wave-length shift have been visual.

B. INSTRUMENTAL

1. *Beckman DK-1 Spectrophotometer.*—We have occasionally measured fluorescent and phosphorescent spectra with the DK-1 spectrophotometer by a technique described by Gemmill.[2] Spectra obtained by this method are subject to considerable error unless the spectral response of the prism-photomultiplier assembly has been calibrated against a standard lamp.

2. *Leiss Monochromator–Red-sensitive Phototube Assembly.*—Emission spectra have been obtained with a Leiss fused quartz monochromator coupled with a DuMont red-sensitive photomultiplier tube, type K 1292, sensitive to 1.3 μ. The photomultiplier signal was piped into a Beckman Model V micromicroammeter, which was reversed to take a negative input signal. Spectral emission intensity was measured as a linear response of the ammeter-galvanometer deflections. The monochromator-photomultiplier-ammeter assembly was calibrated for spectral distribution intensity in relative units against a standard lamp calibrated for us by the National Bureau of Standards. Monochromator slit widths were 0.5 mm. Samples under study were contained in flat-faced Pyrex cuvettes of 4-mm. inside width and were frozen in liquid nitrogen contained in an unsilvered Pyrex Dewar flask. The frozen system was illuminated from one side with a Hanovia Compact xenon arc, and the emission from the opposite side was introduced into the entrance slit of the Leiss monochromator. Judicious use of liquid and glass filters permitted proper separation of exciting and emitted light.

CRITERION 2: LIFETIMES

Since fluorescent lifetimes are of the order of 10^{-8} second in duration, any emission which is apparent after removal of the exciting source, especially when accompanied by a shift toward longer wave lengths relative to the fluorescence emission, is indicative of a metastable-state involvement. Increased lifetimes are obvious visually and may be quantitated from kinetic studies.

CRITERION 3: DECAY KINETICS

The Beckman DK-1 automatic recording spectrophotometer was used for kinetic studies. With the instrument set to record energy, the wave-length drum was set to an appropriate wave length, and the slits were set to maximum width, namely, 2.0 mm. Aqueous solutions of the systems under study were placed in 25 × 200-mm. Pyrex test tubes and immersed initially in a methanol–dry ice bath for prior freezing. Systems studied at 194° K. were permitted to remain in this bath for a minimum of 15 minutes to attain temperature equilibrium. Samples to be studied at 77° K., following freezing in the dry-ice bath, were then transferred to Dewar vacuum flasks containing liquid nitrogen. Samples were allowed to remain here for a minimum of 15 minutes to insure temperature equilibration, following which they were placed for 5 seconds in the unfiltered beam of a Hanovia high-pressure mercury lamp. The glowing sample was then immediately transferred into the lamp-housing compartment of the DK-1 and positioned at the light-entrance port. Following an immediate deflection response of the potentiometer, the magnitude of which depended on the intensity of the afterglow, the decay was tracked automatically, with the recorder chart set to roll at 12 inches per minute. Energy recorded in this manner on the DK-1 was read off the graph in relative linear units. The curves so obtained were examined for fit to kinetic equations.

In making kinetic studies with the DK-1, it should be noted that the instrument itself has a finite decay time. For example, in recording on energy with a light source inducing a 100 per cent energy reading on the potentiometer, shutting off the light causes the potentiometer to "decay" to 0 per cent energy. By running the chart at 12 inches per minute, we were able to obtain an instrument "decay curve." For our instrument this "decay curve" was found to fit a first-order kinetic equation and had a rate constant of approximately 1. Kinetic data obtained with the DK-1, therefore, represent mixtures of two rate constants, that of the instrument and that of the sample being studied. We express our thanks to Dr. William Arnold for deriving an equation for us which permitted the two rate constants to be resolved from mixed kinetic data. Using this equation we have checked the accuracy of our experimentally determined rate constants. The reported rate constants are accurate to within ± 5 per cent; most within ± 2 per cent usually on the low side.

In a communication from the Beckman Instrument Company concerning the use of the DK-1 spectrophotometer for rapid kinetic studies they pointed out that one should be able to determine the time constant (reciprocal of the rate constant) for a phosphorescent decay if it is not less than 10 per cent of the time constant of the DK-1. In our studies no phosphorescences were observed with rate constants exceeding that of our DK-1.

It should be emphasized that in the text figures recording our kinetic results, the ordinates represent phosphorescent emission intensities in relative energy units. No significance is to be attached to the relative vertical positions of the curves on the logarithmic scale. The positions were selected for arbitrary convenience in plotting the data.

A statement accompanying the figures to the effect that the emission for a particular rate study was unfiltered means that the RCA 1P28 photomultiplier tube on the DK-1 was exposed *directly* (the prism system was not traversed) to the glowing sample under study. This technique was possible only at the temperature of dry ice, for at lower temperatures nonspecific emissions interfered. The technique also required extra precautions to exclude outside light from the photomultiplier tube.

CRITERION 4: ACTIVATION ENERGY

Activation energies were measured in the usual way, i.e., from the slope obtained by plotting the log of the rate constant against the reciprocal of the absolute temperature. The temperatures used were those of dry ice (194° K.) and liquid nitrogen (77° K.).

RESULTS

We have obtained and studied delayed emissions for the following compounds (source given): bullock lens, intact and homogenized; tryptophan and tyrosine, H & M Chemical Company; adenine HCl and adenosine, Schwarz Laboratories, Inc.; adenylic acid (muscle), adenosine diphosphate, and adenosine triphosphate, Pabst; desoxynucleic acid (sperm) and ribonucleic acid, Nutritional Biochemical Corporation; progesterone and alpha-estradiol, Ciba; quinine sulfate, Merck; quinidine sulfate, Fisher; acridine orange and acridine yellow, National Aniline Division, Allied Chemical and Dye Corporation. We will now consider the results for the individual materials separately.

1. BULLOCK LENS

For the study of the optical properties of structural proteins, the lens, being devoid of pigments, offers a unique material.[3] Lenses taken from different animals —bullock, rabbit, and various fishes—show a brilliant fluorescence when illuminated with ultraviolet light. When fresh bullock lenses were examined for fluorescence in the dark at room temperature, an intense blue emission was observed which had a lifetime shorter than 10^{-3} second, since no light was observed in our phosphoroscope which had this resolution time. If the lenses were permitted to attain temperature equilibrium at 194° K. (dry-ice temperature), an even more intense emission was exhibited, which persisted, with progressive fading, for about 20 seconds after the exciting light was removed. Such a long-lived afterglow and the shift from fluorescence to phosphorescence on cooling are characteristics of compounds which can undergo singlet-triplet intercombinations. To resolve the question whether the observed phosphorescence was emitted from a triplet state or by a crystal phosphor, we examined the decay kinetics and the activation energy of the emission. Emissions of light by crystal phosphors involve activation energies and are thus strongly temperature-dependent, while the emission decays from triplet excitation states require no activation energy and are temperature-independent. In addition,

the first-order decay kinetics characteristic of triplet emissions is not to be expected with crystal phosphor emissions.

The temperature dependence of the emission lifetime for bullock lens was first qualitatively studied in the following manner. Two Dewar vacuum flasks were placed side by side, one containing dry-ice freezing mixture, the other liquid nitrogen. A test tube holding the bullock lenses was immersed in each. After temperature equilibrium was established, the two test tubes were illuminated side by side for 2–3 seconds with ultraviolet light and then returned to their respective Dewar flasks, and the gradual disappearance of their emissions was observed. The afterglow faded out in both tubes at about the same time, although the temperature difference between the two systems exceeded 110° C. This temperature-independent lifetime strongly pleads for the assumption that the observed phosphorescence comes from a triplet excitation state.

These qualitative observations were quantitatively corroborated from kinetic studies carried out as described under the technique for Criterion 3, decay kinetics. Figure 1 depicts the results. It is to be noted first that the curves fit a first-order kinetic equation, i.e., the decay rate is a function only of the concentration of the residual metastable, triplet, species. Second, since the decay-rate constant is temperature-independent, the activation energy is negligible, and we have further evidence for triplet-state involvement.

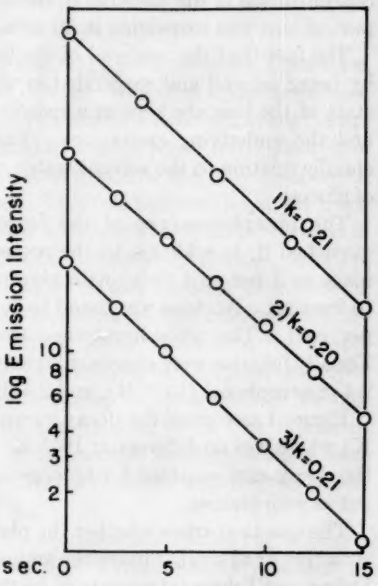

FIG. 1.—Phosphorescent decay kinetic data for lens systems. k = rate constant. *1*, intact ox lens, T = 194° K., emissions unfiltered; *2*, intact ox lens, T = 77° K., emission measured at 420 μ; *3*, homogenized ox lens (diluted 1 to 30 with water), T = 77° K., emission measured at 420 mμ.

The structural protein of the lens forms a fairly solid gel in vivo and can thus be expected to be fixed in space. This matrix should also impede the diffusion of dissolved protein molecules. This assumption must hold even more strongly for a frozen system. The change from fluorescence to phosphorescence with cooling can thus hardly be ascribed to dimerization or to higher polymerization of protein molecules but rather must be attributed to a change in the solvent and its relation to the protein.

If the lenses are minced in a Latapie mincer and suspended in 30 volumes of water (our lens homogenate), the suspension shows no striking fluorescence and no phosphorescence at 194° K. The same is true for less dilute suspensions. If, however, the suspensions are cooled further to 77° K., they emit a phosphorescence with an afterglow duration indistinguishable from that of the intact lens.

It was noted earlier that the addition of glucose to aqueous solutions of fluorescent substances in many instances enhances the phosphorescence of the frozen state.

especially at 194° K. This effect was ascribed to a more intimate contact of the luminescent species with water, glucose disfavoring a sharp separation of water crystals from the solute. This effect was observed also with the lens suspension. If the suspension contained 0.5 per cent glucose, freezing to 194° K., with subsequent illumination, caused the appearance of a strong blue emission resembling that of the intact lens and persisting for about half a minute after removal of the illumination. This afterglow was fairly independent of glucose concentration, being equally strong at 0.25 per cent and at 5 per cent. The light intensities were, under the conditions of our experiment, also independent, within wide limits, of the concentration of the suspension, the afterglow being about equally strong whether 1 part of lens was suspended in 30 or in 300 parts of water.

The fact that the material of the lens loses its fluorescence and phosphorescence by being minced and suspended in water indicates that the proteins in the native state of the lens are kept in a specific condition which favors these light emissions and the underlying excitations. This condition in all probability involves some specific relation to the solvent water, which relation can be restored by the addition of glucose.

The phosphorescence of the frozen (194° K.) lens suspension was strongly quenched if, in addition to the presence of 0.5 per cent glucose, the solution was made to 3 per cent (v/v) with glycerol or 0.25 per cent (v/v) with methanol prior to freezing. Acetone was found to be inactive even at a concentration of 8 volumes per cent. The phosphorescence was also insensitive to the action of oxygen. Equally inactive were classes of "quenchers" such as KI (10^{-3} M), NaSCN (10^{-3} M), 2,4-dinitrophenol (10^{-5} M), and 2,4-dinitronaphthol (5×10^{-6} M).

Figure 1 also gives the decay curve for homogenized lens phosphorescence at 77° K., which was no different at 194° K. In addition, it should be noted that at 77° K. the decay-rate constant for homogenized lens phosphorescence was the same without as with glucose.

The question arises whether the phosphorescence of the lens is due to some superstructure or to smaller units like amino acids making up the molecule. The paper of Debye and Edwards[4] appears to be the first systematic attempt to examine protein phosphorescence. They observed that most proteins emitted a brilliant blue phosphorescence at liquid-nitrogen temperature. The visible spectrum was found to be pH-dependent, the protein phosphorescence from an alkaline medium exhibiting a minor maximum at 418 mμ and an intense maximum at 440 mμ. In an acid medium the results were less consistent, the main maximum appearing at 417 mμ. The phosphorescent spectra of tyrosine and tryptophan were found to lie in the same spectral region as the protein phosphorescence. Debye and Edwards found the amino acid phosphorescent emission decays to be exponential and suggested that the exponential decay phosphorescence of proteins was due to their content of tyrosine, tryptophan, and possibly phenylalanine. From their finding that the phosphorescent decay was temperature-independent, they felt that an electronic transition was involved. Grossweiner,[5] using flash photolysis techniques, has complemented this work of Debye and Edwards and has presented spectra for the metastable states of ovalbumin, tyrosine, phenol, tryptophan, and indole. The wave-length shift, temperature independence, and metastable absorption spectra obtained by these workers suggested triplet-state involvement. Grossweiner further concluded that

the metastable absorption spectrum for ovalbumin principally represented the sum of contributions from tyrosine and tryptophan, with negligible effects from the peptide linkages.

The lens phosphorescence which we have observed (see Fig. 1) appears to have a slightly longer mean lifetime (5 seconds) than the protein phosphorescence studied by Debye and Edwards (approximately 3 seconds). In addition, we have observed slight differences in the kinetic data for amino acid phosphorescences when compared with the data of Debye and Edwards, but these are probably differences in degree only and not in kind arising from differences in technique.

2. AMINO ACIDS

The following amino acids were tested: *dl*-alanine, *l*-glutamic acid, *l*-histidine, *dl*-phenylalanine, *l*-tyrosine, and *dl*-tryptophan. None of these in aqueous solution showed any striking luminescence on visual observation under the ultraviolet lamp, frozen or unfrozen. Of these amino acids it might be anticipated from absorption spectral data that tryptophan and possibly tyrosine would be the only compounds which could possibly emit a visible fluorescence. This fact becomes apparent when one examines the fluorescent spectral data for the aromatic amino acids reported in the recent publications of Bowman, Caulfield, and Udenfriend,[6] Sprince, Rowley, and Jameson,[7] and Teale and Weber.[8] Phenylalanine and tyrosine emissions lie exclusively in the ultraviolet, while tryptophan emission, with a maximum in the near-ultraviolet, has a long-wave-length tail which "spills" over into the visible out to about 440 mμ. For this reason, therefore, a tryptophan solution of proper dilution, upon exposure to an unfiltered ultraviolet lamp, emits a distinct, though weak, violet fluorescence easily observable with the unaided eye. The failure to observe any appreciable fluorescence in a 0.01 *M* tryptophan solution, while a solution of 10^{-4} *M* emits a distinct violet light, indicates that the negative results at higher concentration may be due to self-quenching.

This emission picture changed markedly for the aromatic acids when the solutions were made to 0.5 per cent in glucose prior to freezing. Tyrosine and tryptophan in 10^{-3} molar concentration gave short and prolonged afterglows, respectively. These emissions were of considerable intensity. The behavior of tryptophan appeared similar to that of the lens, being blue and lasting for about half a minute. The wave-length shifts for these two compounds in going from fluorescence to phosphorescence were visually apparent, the colorless emission of tyrosine shifting to blue and the violet emission of tryptophan shifting to blue.

The marked enhancement of phosphorescence in the frozen state displayed by many compounds in the presence of glucose (other sugars were equally as effective) motivated us to examine this effect in more detail. Studies were made with tryptophan as the luminous species.

It was found that the phosphorescent decay-rate constant for tryptophan-glucose systems in ice was practically independent of temperature[9] and tryptophan concentration. In addition, we have observed an influence of glucose concentration on the phosphorescent emission intensity. When the tryptophan concentration was held constant and the glucose concentration varied, the emission intensity was observed to pass through a maximum, while the decay-rate constant remained essentially unchanged. A preliminary examination of this effect made with a tryptophan con-

centration of 5×10^{-4} molar resulted in a maximum phosphorescent intensity at a glucose concentration of 0.27 per cent. Table 1 summarizes the results for the slight influence of temperature and tryptophan concentration on the phosphorescent decay kinetics.

Substitution of the rate data from Table 1 for the 10^{-3} molar tryptophan system into the equation,

$$\log \frac{k_2}{k_1} = \frac{\Delta H_a}{2.303R}\left(\frac{T_2 - T_1}{T_2 T_1}\right),$$

gave an activation energy of only 76 calories. In this equation k_2 and k_1 are the rate constants at the high (T_2) and low (T_1) temperatures, respectively; ΔH_a is the activation energy; and R is the gas constant.

Figure 2 illustrates the influence of temperature on the phosphorescent decay-rate constant for a 10^{-4} molar tryptophan concentration system containing 1 per cent glucose. It should be observed that the tryptophan phosphorescence was insensitive to oxygen.

TABLE 1

THE INFLUENCE OF TEMPERATURE AND TRYPTOPHAN CONCENTRATION ON THE DECAY-CONSTANT FOR TRYPTOPHAN PHOSPHORESCENCE*

TRYPTOPHAN CONCENTRATION (MOLES PER LITER)	RATE CONSTANT, k		TRYPTOPHAN CONCENTRATION (MOLES PER LITER)	RATE CONSTANT, k	
	194° K.	77° K.		194° K.	77° K.
5×10^{-3}	0.40	0.39	10^{-5}	0.26	0.17
10^{-3}	0.27	0.20	10^{-6}	0.24	0.17
10^{-4}	0.21	0.18			

* The glucose concentration was held constant at 0.25 per cent. Rate data were obtained with the spectrophotometer set at 420 mμ and with 2.0-mm. slit widths. Systems were contained in 25 × 200-mm. Pyrex test tubes and, following a minimum of 15 minutes at the proper temperature to allow temperature equilibration, were illuminated with the unfiltered beam of a high-pressure Hanovia mercury arc for 5 seconds.

Figure 3 contains the phosphorescent decay kinetic data of the tyrosine-glucose system. The intensity of the emitted light in the absence of glucose was too low to permit its measurement by the technique used in this study. At 194° K., even in the presence of glucose, the intensity of the emitted light was so low that it was difficult to obtain a satisfactory decay curve, and the reported rate constant should be considered accurate within ± 5 per cent. We have made no effort to obtain kinetic data for phenylalanine.

Wave-length shifts, long-lived afterglows, first-order decay kinetics, and negligible temperature dependence of the rate constants may be presented as presumptive evidence that tyrosine and tryptophan afterglows are emitted from a metastable triplet state.

3. ADENINE, ADENOSINE, ADENOSINE PHOSPHATE, ADENOSINE DIPHOSPHATE, AND ADENOSINE TRIPHOSPHATE (ATP)

Euler, Brandt, and Neumüller[10] and Stimson and Reuter[11] presented fluorescent data for several purines and pyrimidines and their derivatives and gave the color of their emissions as a function of pH. Since we have been interested in the energy configuration of ATP in relation to its possible functional participation in the energy transformations in which the molecule participates, we have limited our study to the purine adenine and its derivatives.

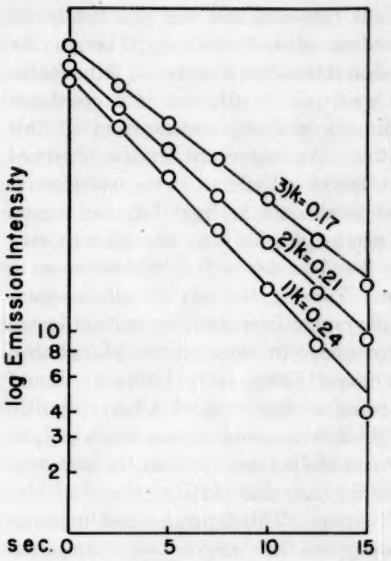

FIG. 2.—Influence of temperature on the phosphorescent decay rate constant, k, for tryptophan-glucose system; tryptophan = 10^{-4} M, glucose = 1 per cent. *1*, system *approaching* temperature equilibrium at 194° K.; *2*, system at temperature equilibrium at 194° K.; *3*, system at temperature equilibrium at 77° K., emission measured at 420 mμ.

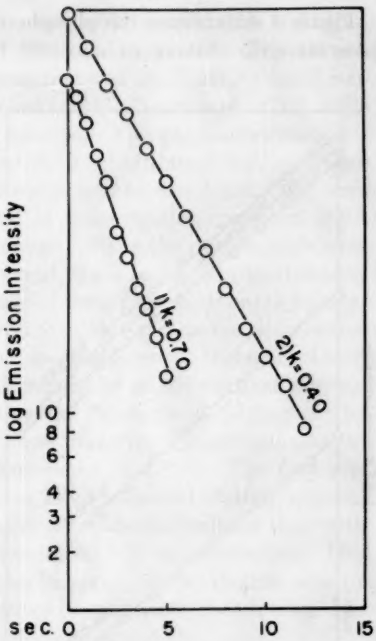

FIG. 3.—Phosphorescent decay kinetic data for tyrosine-glucose systems. k = rate constant. *1*, 10^{-3} M tyrosine + 0.5 per cent glucose, T = 194° K., emission unfiltered; *2*, 4.4×10^{-4} M tyrosine + 0.3 per cent glucose, T = 77° K., emission measured at 420 mμ.

In view of the fact that the absorption bands of the adenine compounds lie rather deep in the ultraviolet, it might be anticipated that only slight or no visible fluorescence would be observable from them upon proper excitation. Stimson and Reuter[11] reported a faint green fluorescence for adenine sulfate in strong alkali and a faint blue emission in weak alkali. They observed no emission for adenylic acid solutions at any pH. In our work we have never observed a visible emission from adenine, adenosine, or any of the phosphate derivatives at any pH at room temperature. Adenine compounds of 0.01–0.0005 M concentrations, when frozen to 194° K., displayed a very faint blue short-lived afterglow. This emission could usually be enhanced in intensity by the addition of glucose to 0.5 per cent prior to freezing. ATP, for example, with glucose added, displayed an intense afterglow lasting for about 15 seconds after the exciting light was removed. ATP, in contrast to tryptophan, displayed no self-quenching in 0.01 M concentration. This may have biological significance, especially in muscle, where ATP exists in rather high concentration. While the ATP phosphorescence was insensitive to KI, NaSCN, 2,4-dinitrophenol, 2,4-dinitronaphthol, and acetone (8 per cent v/v) it was abolished by 5 per cent (v/v) glycerol and 0.1 per cent (v/v) methanol at 194° K. These later quenching effects were not observed, however, at 77° K.

Figure 4 summarizes the phosphorescent decay kinetics for all the adenine systems studied. Kinetic data at 194° K. were obtained for ATP only and indicated that the rate constant was practically independent of temperature. Though the emission intensities were found to be markedly sensitive to pH, the rate constants remained essentially independent of this variable. An important feature observed in studies of pH effects on the emission intensities was the finding that the amino nitrogen at position 6 on the adenine ring must be dissociated for luminescence to occur. Varying the pH of adenine-compound systems from 2 to 7 by units of 1 gave a progressive increase in the phosphorescent intensity from nil to brilliant. Since, according to the work of Alberty, Smith, and Bock,[12] the amino group has a pK_a of approximately 4, we visualize the increased alkalinity as dissociating the "adeninium" cation. This dependence of luminous intensity on the degree of dissociation of an amino group is not a unique finding. Ley and von Engelhardt[13] noted that, whereas the aniline molecule was fluorescent, the anilinium cation was nonfluorescent. By contrast, Ley and Gräfe[14] and Dickson[15] found that the blue fluorescence of alpha- and beta-naphthylamine was not lost by proton addition but, instead, was shifted into the ultraviolet, with, however, a considerable diminution in intensity. Lewis and Kasha[16] have given phosphorescent emission data for aniline and alpha- and beta-naphthylamine, but we have found no reports on the phosphorescence of the anilinium cation or of the alpha- or beta-naphthylammonium cations.

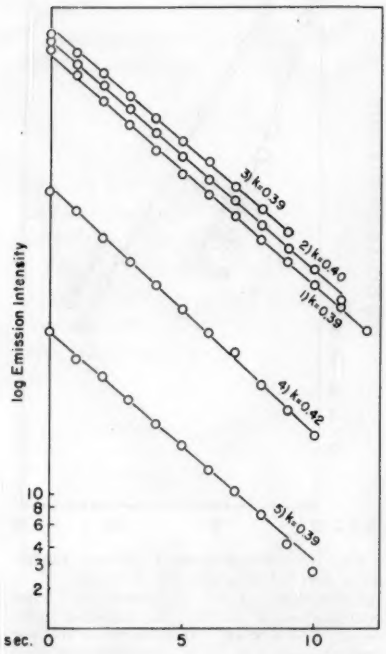

FIG. 4.—Phosphorescent decay kinetic data for adenine (*1*), adenosine (*2*), adenosine-5′-phosphate (*3*), adenosine-5′-diphosphate (*4*), and adenosine-5′-triphosphate (*5*). All concentrations 0.001 *M*; pH's adjusted to 5 with acetate buffer prior to freezing. T = 77° K. All emissions measured at 420 mμ. k = rate constant.

For the adenine derivatives studied, therefore, the wave-length shifts, i.e., from no visible emission to an intense blue afterglow, the long lifetimes, and the first-order kinetics plead for triplet-state participation in the emissions observed for these compounds.

4. NUCLEIC ACIDS

Nucleic acids show a long-lived phosphorescence which might be due to the collective action of the column of cyclic structures constituting, according to the model of Watson and Crick,[17] the core of the molecule. The central cyclic compounds would thus, according to this assumption, be analogous to Scheibe's pseudoisocyanine dyes,[18] which polymerize to columns in the shape of "money-rolls" through which excitons are freely propagated by resonance.

The phosphorescent light emission from aqueous solutions of DNA and RNA preparations, 0.1–1.0 per cent, frozen to 194° K., could be prolonged by the addition of 0.25–4 per cent glucose. This prolongation was abolished by small concentrations of glycerol (3 per cent, v/v) or methanol (0.25 per cent, v/v), while acetone (16 per cent, v/v) was found to be inactive. The phosphorescence was found to be insensitive to KI, 2,4-dinitrophenol, 2,4-dinitronaphthol, salicylate (0.002 M), or pyrrolazote (2×10^{-4} M). Morphia and aconitine (10^{-3} M) were equally inactive, while colchicine in 2×10^{-4} M concentration quenched DNA strongly, the effect increasing somewhat on storage. Since the nucleic acids were present in rather high concentration, 0.5–1 per cent, there was a disproportionality between the molarity of the alkaloid and the mass of the nucleic acids or the number of nucleotides building the nucleic acid molecule. This pleads for a collective action of the nucleotides in the production of phosphorescence. Scheibe[19] found the energy transmission in his dye polymers decreased by relatively small concentrations of hydroquinone, the conduction within the "money-rolls" being cut by the interposition of a "dud," a hydroquinone molecule. Hydroquinone at 0.001 M also strongly quenched the phosphorescence of DNA. The first preliminary experiments repeated with high-polymer DNA indicated that the quenching action of colchicine was about ten times stronger with this material than with the low-polymer DNA, the alkaloid being active in 10^{-5} M concentration. This concentration is near the level which is effective in vivo. If the alkaloid acts by cutting the energy transmission through the row of central bases, then it should be more active the longer the molecule.

The carcinogen benzpyrene also showed a fairly strong quenching activity. In these experiments the carcinogen was dissolved in acetone (0.1 per cent). One-tenth of a milliliter of this solution added to 5 ml. of 0.5 per cent DNA almost completely eliminated the long afterglow enhanced by glucose. In this instance, too, the action seemed to be more marked when high-polymer DNA was used in the experiment. Benzpyrene, as is generally known from the work of Brock, Druckrey, and Hamperl,[20] emits a blue light when molecularly dispersed and a yellow-green light in colloidal emulsion when illuminated by ultraviolet light. This can be strikingly demonstrated by adding a drop of acetone solution of benzpyrene to water. The resulting colloidal solution emits a yellow-green fluorescence which changes dramatically to blue if the solution is shaken up with a little solid caffein. The purine forms a complex with the carcinogen, bringing it into molecular dispersion. Analogous behavior can be demonstrated with DNA, indicating that the carcinogen complexes with the bases of the nucleic acids. This might have been anticipated also from the work of Booth and Boyland,[21] who demonstrated that the variably carcinogenic bicyclic dibenzacridines formed well-defined crystalline complexes with purines and nucleic acids.

Figure 5 depicts the phosphorescent decay kinetic results as measured for DNA and RNA at 77° K. Decay kinetics are not reported for these compounds at 194°K., for the emission intensities were too low to be recorded by the technique here employed. The rate constants given should be considered only as indicating the order of magnitude of the decay rate, for the purity of these preparations was uncertain. The rate constant for DNA appears to be complex, and until highly purified preparations of reasonably well-known polymer composition are avail-

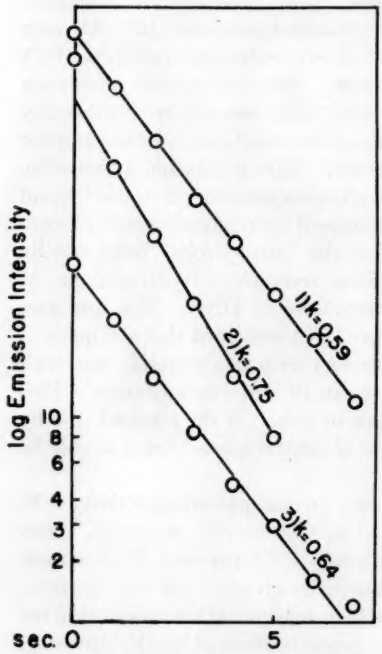

FIG. 5.—Phosphorescent decay kinetic data for desoxyribonucleic acid and ribonucleic acid. $T = 77°$ K. k = rate constant. *1*, 1 per cent DNA + 0.25 per cent glucose, 480 μ; *2*, 1 per cent DNA, no glucose, 520 mμ; *3*, 1 per cent RNA + 0.25 per cent glucose, 400 mμ.

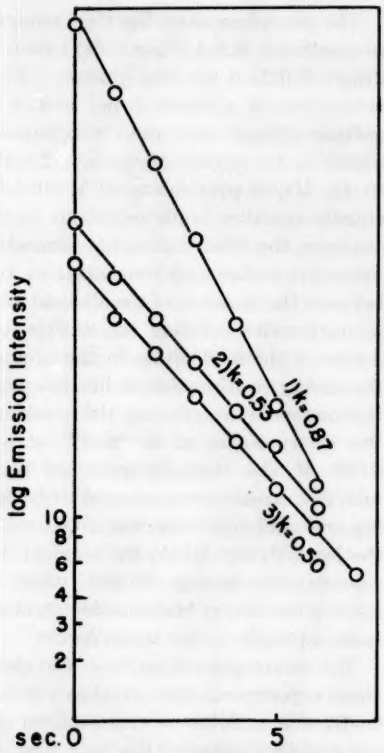

FIG. 6.—Phosphorescent decay kinetic data. *1*, quinidine-SO_4, 10^{-4} M aqueous solution + 0.25 per cent glucose, green emission measured at 510 mμ, $T = 77°$ K.; *2*, progesterone, 7.3×10^{-4} M in 95 per cent ethanol + 0.5 per cent glucose, blue emission measured at 420 mμ, $T = 77°$ K.; *3*, alpha-estradiol, 10^{-3} M in 95 per cent ethanol, no glucose, violet emission measured at 410 mμ, $T = 77°$ K. k = rate constant.

able, we will restrict our comments to one observation, namely, that while a 0.1 per cent DNA solution (glucose present) displayed a blue phosphorescence, a 1 per cent DNA solution (also in the presence of glucose) gave a green phosphorescence. We have noticed a somewhat analogous phenomenon with adenosine. Whereas adenosine in the absence of glucose emitted a distinct green phosphorescence, in the presence of glucose the emission reverted to the blue characteristic for adenine and the adenosine phosphate derivatives. The effect was most marked at low pH values, and none of the other adenine compounds showed the effect. Therefore, the difference observed in the rate constant for DNA may reflect a mixture of these two emissions. The data predict that the green emitting system, if real, will be found to have the higher rate constant. No such wave-length shifts have been observed for RNA.

First-order kinetics and increased lifetimes are the only criteria we have obtained so far for the nucleic acids which suggest triplet-state involvement.

5. MISCELLANEOUS

Figure 6 contains rate data for quinidine sulfate, progesterone, and alpha-estradiol phosphorescences, all measured at 77° K. and at the wave lengths given. Quinine sulfate gave practically the same rate constant as quinidine sulfate, and glucose addition was necessary for both compounds in order to obtain sufficient luminous intensity to permit decay curves to be run. The emission was a distinct green in both instances and of rather intense brightness, though of very short duration. The blue emission of progesterone and the violet emission of alpha-estradiol, though of low intensities, could both be measured without glucose addition; in fact, glucose addition had little influence on their emission intensities. It should be noted that the solvent for the hormones was 95 per cent ethanol, not water. The very low emission intensities at 194° prevented rate-constant measurements by the technique used in this study.

Quinine and quinidine sulfate fluoresced blue, while the phosphorescence was green. We have measured the fluorescence of alpha-estradiol (unpublished) and found it to be exclusively ultraviolet, with a maximum at approximately 310 μ. We were unable to obtain any indication of progesterone fluorescence. With these compounds, therefore, wave-length shift (with the possible exception of progesterone), first-order kinetics, and increased lifetimes represent the criteria suggestive of triplet-state involvement.

6. DYES

In Figure 7 are presented a few preliminary kinetic studies made for the dyes acridine yellow and acridine orange. The break in rate curves at the higher dye concentration and elevated water content suggest that polymer involvement may influence the rate constant. Zanker[22] has made a detailed study on the influence of solvent, concentration, temperature, and pH on the absorption and emission characteristics of acridine orange that may prove to be pertinent here.

We have measured the following emission shifts for these two dyes in going from fluorescence to the phosphorescent state. Acridine yellow fluoresced at 511 mμ and phosphoresced with a maximum at 614 mμ. Acridine orange gave a fluorescence band with a maximum at 530 mμ and a phosphorescence-band maximum at 660 mμ. These wave-length shifts, first-order kinetics, and long-lived afterglow, we cite, therefore, as evidence for triplet-state participation in the phosphorescence of these dyes.

7. WATER

A finding which we can mention only briefly at this time was an observation concerning the emission from ice following ultraviolet excitation.[23] The "afterglow" was not visible in an undarkened room, and we have made no attempt to visualize it with dark-adapted eyes. The intensity appeared greatest when the emission from ice at 77° K. was permitted to impinge unfiltered directly onto the RCA 1P28 photomultiplier tube set electronically at maximum sensitivity. Precautions were always taken to insure that this ice emission did not interfere with the

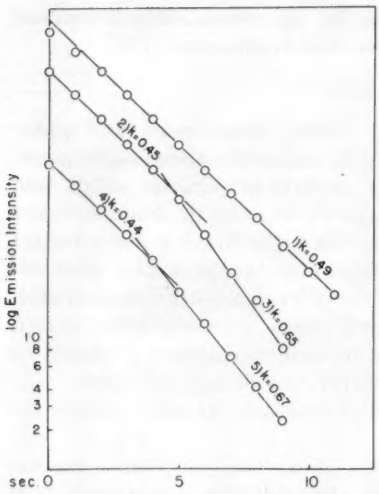

FIG. 7.—Phosphorescent decay kinetic data for the dyes acridine yellow and acridine orange. $T = 77°$ K. k = rate constant. *1*, 10^{-4} *M* acridine yellow in 90 per cent ethanol + 10 per cent water (v/v), emission measured at 550 mμ; *2–3*, 5×10^{-4} *M* acridine yellow in 50 per cent ethanol + 50 per cent water (v/v), emission measured at 530 mμ; *4–5*, 5×10^{-4} *M* acridine orange in 50 per cent ethanol + 50 per cent water (v/v), emission measured at 600 mμ.

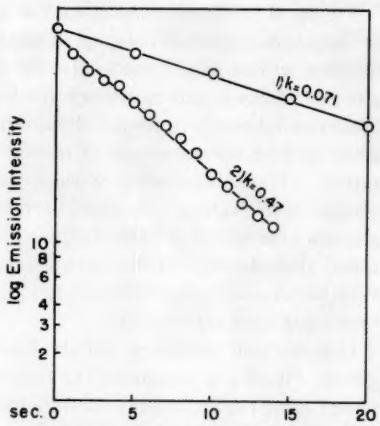

FIG. 8.—Phosphorescent decay kinetics for the unfiltered afterglow from ice at 77° and 194° K. The ice was frozen around the outside of a hollow aluminum bar (freezing mixtures inside) and exposed for 5 seconds to the unfiltered beam of a high-pressure mercury lamp prior to the kinetic run. k = rate constant. 1 = 77° K., 2 = 194° K.

kinetic measurements. Rate data for this emission at 77° and 194° K. are given in Figure 8. These results were fairly reproducible. It is interesting that the decay appears to obey first-order kinetics and has a rather low activation energy. Unfortunately, the intensities were too low to permit us to obtain reliable information about their spectral distribution. The rate constant, however, appeared to be about the same whether the emission was permitted to traverse a Corning 5860 band-pass filter (maxinum wave length passed at 360 mμ) or a Corning 3486 cutoff filter, cutting out light *below* 510 mμ.

DISCUSSION AND SUMMARY

Fluorescent and phosphorescent spectral data, kinetic studies (including order of reaction), lifetimes, and activation-energy considerations have been presented for a wide variety of biological compounds and the dyes acridine yellow and acridine orange. Results were obtained which we submit as evidence that electronic transitions involving singlet-triplet intercombinations are functional in these compounds.

The marked optical changes observed in freezing *aqueous* solutions, namely, the shift from fluorescence to phosphorescence, appear to be a universal property of fluorescent materials.

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[23] Leonard I. Grossweiner and Max S. Matheson (*J. Chem. Phys.*, **20**, 1654–1655, 1952; **22**, 1514–1526, 1954) have reported observing an X-ray-induced fluorescence from ice at low temperatures. The emission at −183° C. had an almost symmetrical band with a peak at 385 μ. They observed no phosphorescence whatever. That energy was trapped, however, was made evident from glow-curve studies following X-ray excitation. The difference between their activation energy (0.25 ev.) and ours (0.02 ev.) indicates that different mechanisms are involved in "trapping" the energy. This would be expected, since energy was stored in the one instance by X-ray excitation and in the other instance by ultraviolet light. We have made no study of the influence of oxygen on the emission, and dissolved oxygen was not excluded prior to freezing. Grossweiner and Matheson observed a suppression of thermoluminescence by oxygen.

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*REPRESSED AND INDUCED ENZYME FORMATION: A UNIFIED HYPOTHESIS**

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A specific antagonism, termed "enzyme repression," of the formation of the biosynthetic enzyme acetylornithinase in *Escherichia coli* has recently been described.[1] The repressing agent was found to be arginine (or a substance related to arginine), which is the "end product" of the biosynthetic sequence involved. Enzyme repression by an exogenously available end product (combined with

anabolic utilization of this product) represents a highly effective regulatory device, since it permits the cell involved to curtail superfluous enzyme production. Enzyme repression by an endogenously produced substance can provide a feed-back arrangement that governs, in line with cellular demands, the flow of metabolites through the pathway leading to this substance. Accordingly, enzyme repression is a control mechanism that complements enzyme induction: in either case, the cell tends to form enzymes when they are needed and tends not to form enzymes when they are not needed. It has been pointed out that repressibility appears to be a widespread property of enzyme-forming systems, which presumably was positively selected in the course of evolution. The possibility that enzyme repression plays a role in the development or differentiation of higher forms of life has also been briefly mentioned. The present communication is concerned with a hypothesis of enzyme induction and repression.

"Small-Molecule" Control of Enzyme Formation.—As previously discussed, repression and induction appear to be separable from the process of enzyme formation per se; in particular, there are good grounds for the view that induction is not a *sine qua non* of enzyme formation. It is for this reason that repression and induction are best pictured as control mechanisms rather than as inherent features of enzyme biogenesis.

The following small-molecule control situations with respect to enzyme formation are conceivable: (i) indifferent enzyme synthesis (no control); (ii) induced enzyme synthesis; and (iii) repressed enzyme synthesis. One and the same enzyme-forming system may be both inducible and repressible,[2] or may be neither. Again, a given enzyme-forming system may be inducible but not repressible, or repressible but not inducible.

Repression and induction can thus be considered to have a functional correspondence. In addition, these processes may well depend on corresponding molecular mechanisms. The question then arises how a relatively small molecule, such as an inducer or a represser, can specifically modify the formation of a particular protein of large molecular weight.

A Unified Hypothesis of Induction and Repression.—From their relative specificity it seems reasonable to assume that induction or repression depend, respectively, on an interaction between inducer or represser (or their "active" derivatives), on the one hand, and a macromolecule, presumably of template nature, on the other. Such interaction either may involve the template and the "regulator" (inducer or represser) or may involve template, template product, and regulator. In line with current assumptions,[3] this template may be one immediately involved in the production of enzyme protein.

Since action of the regulator—in particular, of the inducer—does not appear to be a *sine qua non* of enzyme formation,[1] the cell is thought to have, prior to contact with the inducer, the essential information required for enzyme structure. Accordingly, the inducer would seem to act not by furnishing a prototype for the configuration of the enzyme molecule but, rather, by improving the performance of the template. Thus the inducer would assist the template catalyst, and may be considered a "promoter." In contrast, a represser may be compared to a catalyst "poison."

It is proposed that inducers and repressers act by affecting the rate of dissociation

of a template product from its template. Repression could then be the result of binding of newly formed enzyme protein to its site of synthesis through the agency of the represser involved. Induction may reflect the neutralization of a binding effect which, in the absence of the inducer, would tend to hold the nascent protein near its template. It is considered that rapid removal of template product from template will accelerate enzyme formation, while occupation of a template by its product will prevent the template from functioning in further enzyme synthesis. It is not necessary to assume that the represser causes binding of enzyme to template by acting as a "bridge"; it is possible that the represser causes a change in macromolecular configuration which results in binding. Similarly, an inducer may act either through the local "neutralization" of a bridge group or through an effect on macromolecule configuration.

Regulators may be expected to show greater or lesser tendencies to depart from their locales of action. Thus, in line with Pollock's observations,[4] the inducer of penicillinase (in contrast to inducers of β-galactosidase) appears to be tightly held at the induction site.

In the present view of regulator action, the separation of template product from template is regarded as a more or less well-defined, single event. However, this view is not necessarily in conflict with a (simultaneous) multiple partial functioning of a template, such as is envisaged by Dalgliesh.[5] In "two-dimensional" protein synthesis,[6] however, one wonders to what extent such partial functioning of "one-dimensional" templates is likely to occur.

The hypothesis here proposed, which will be referred to as the "regulator hypothesis," implies that all enzymes, be they constitutive or adaptive, inducible or repressible, are synthesized in the same general manner but may differ from case to case in susceptibility to regulators. This picture differs from Cohn and Monod's[7] "generalized induction hypothesis" (cf. Vogel[1]).

The Regulator Hypothesis and the "Final Shaping" of Enzymes.—In its general form, the regulator hypothesis is concerned with the dissociation of a template product from its template. Let it now be assumed that the template product is the nascent enzyme protein and that the regulator acts in a region that corresponds to the dynamic site of the "finally shaped"[8] (see below) enzyme molecule. If so, the regulator would be expected to be in close physical contact with the nascent dynamic site at the very moment that the template product separates from the template. Such contact may well be of considerable consequence for the fruitful final shaping of the enzyme molecule, particularly in the folding of the nascent enzyme protein or in the aggregation of template products. A directed folding process in protein biogenesis has been considered by previous authors, especially in connection with antibody formation (Pauling-Haurowitz-Mudd hypothesis; cf. Haurowitz[6]). In principle, the importance of the final shaping process has been anticipated—for example, in the specific-precursor postulates of Monod[9] and of Pollock.[4]

Lipmann[10] has recently drawn attention to the final shaping of proteins as distinct from the emergence of a defined sequential arrangement ("patternization") of component amino acids. While, as suggested above, final shaping may be affected by regulators, this process is considered to be capable of occurring in the absence of regulators. For instance, appropriate folding of nascent enzyme protein could

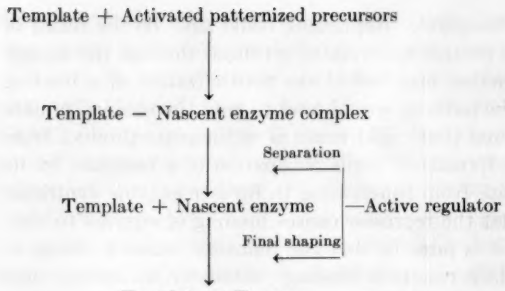

FIG. 1.—Scheme of regulator action

be a spontaneous consequence of the patternization involved.[10] The possible role of regulators in influencing (*a*) the separation of template product from template and (*b*) the final shaping is presented schematically in Figure 1.[11]

Comments on the Regulator Hypothesis.—The regulator hypothesis is generally consistent with and supported by the available data on enzyme induction and enzyme repression. This hypothesis readily accounts for the observation (cf. Pollock[4]) that inducible enzymes usually show a "basal" enzyme level in the absence of added inducers. The basal level would correspond to the rate of enzyme production that the cell is capable of achieving without the benefit of the promoting effect of the inducer.

The emergence of mutants in which an originally inducible enzyme has become constitutive (cf. Monod and Cohn[12]) can be visualized, at least in some cases, as involving a template alteration such that the corresponding template product is enabled to dissociate rapidly enough from its site of formation without aid from a regulator substance; in other words, there would be a change from inducible to indifferent enzyme synthesis.

The present hypothesis is also in harmony with other results, including the finding[13] that there is no necessary relationship between the properties of a given substance as an inducer (or a represser), on the one hand, and a substrate or a complexant, on the other. Again, the regulator hypothesis is in accord with the further findings that different inducers can evoke enzymes of indistinguishable specificity[12] and that an enzyme produced under conditions of repression has been found not to differ detectably from the corresponding nonrepressed enzyme.[1]

A dual regulator function, such as that illustrated in Figure 1, appears to be consistent with available results on induced enzyme formation, including those of Cohn and Torriani.[14] These authors have considered the possibility that the β-galactosidase and the immunochemically related (but enzymatically inactive) Pz protein of *E. coli* are synthesized independently at the same site. In line with this possibility, the increased rate of β-galactosidase formation and the decreased rate of Pz formation observed upon induction may well involve the above-mentioned dual regulator effect. However, since it has not been definitely established that β-galactosidase and Pz actually are produced at the same site, other explanations for the relation between these two proteins remain to be considered.

The Regulator Hypothesis and Other Theories of Enzyme Formation.—In a recent review on theories of enzyme adaptation, Mandlestam[15] has aptly commented on the unusually high ratio of theories to facts in this field; he has also pointed out, however, that it is unlikely that this subject would have advanced so rapidly if the theories had not been formulated. A comparison of the regulator hypothesis with some earlier concepts might therefore not be without some value.

The major theories of induction can be classified as (I) those that depend on some kind of combination of inducer with (finally shaped) enzyme (for either equilibrium displacement or enzyme stabilization or template-like action) and (II) those that do not.

Group I includes Yudkin's[16] mass-action theory and Mandlestam's[17] extended-mass-action theory; Spiegelman's[18] plasmagene theory and Monod's[9] specific-precursor theory; and Campbell and Spiegelman's template-inducer-enzyme complex theory (cited by Spiegelman and Campbell[3]), in which such a triple complex is considered to be the only structure that can effectively and rapidly lead to enzyme synthesis. Group II contains Pollock's[4] organizer hypothesis (which has been extended by Cohn and Monod[7]), Monod and Cohn's[12] inducer-prototype hypothesis, and the present regulator hypothesis.

Arguments against those theories in Group I which invoke a binary complex of (finally shaped) enzyme and inducer have been presented by Monod *et al.*[13] These authors showed that, for β-galactosidase of *E. coli*, inducer function is separable from substrate or complexant functions. Their arguments do not apply to a triple complex, such as is contemplated by Campbell and Spiegelman. The triple-complex hypothesis was advanced primarily to account for "long-term adaptation" in yeast.[3] Since, however, other possible explanations[19] of long-term adaptation have not been excluded, one is not forced to assume that a given finally shaped enzyme participates in the synthesis of its own molecular species. It is also worthy of note that the above-mentioned results of Monod *et al.*[13] do not provide evidence against a complex involving an inducer and the corresponding nascent enzyme (which may be similar to, but not identical with, the finally shaped enzyme in affinity for complexants and in enzymatic activity).

Group II, which includes hypotheses that do not invoke an active participation of finally shaped enzyme in the production of its species, can be subdivided into the organizer and inducer-prototype hypotheses, on the one hand, and the regulator hypothesis, on the other. The inducer-prototype hypothesis assumes that the steric configuration of an enzyme's active site is directly derived from the inducer.[12, 20] Similarly, the organizer hypothesis has been interpreted[7] as implying that the characteristic pattern of an enzyme's dynamic site is imposed by a co-organizer ("active" inducer). Arguments against such a prototype or co-organizer function of the inducer have recently been discussed.[1] In the regulator hypothesis, the assumption of prototype function is avoided.

Summary.—A hypothesis (regulator hypothesis) has been proposed to account for induced and repressed enzyme formation, which are viewed as analogous phenomena in terms of function and of mechanism. Regulators (inducers or repressers) are thought to act, respectively, by promoting or impeding the separation of template product (nascent enzyme protein) from template. An effect of regulators on the final shaping of enzyme molecules is also contemplated. Regulator action is pictured as a control mechanism rather than as a *sine qua non* of enzyme biogenesis.

* This work was aided by a contract between the Office of Naval Research, Department of the Navy, and Yale University, and by the Damon Runyon Memorial Fund.

[1] H. J. Vogel, in W. D. McElroy and B. Glass (eds.), *The Chemical Basis of Heredity* (Baltimore: Johns Hopkins Press, 1957), p. 276. In this paper other results best interpreted as enzyme repres-

sion are cited. A convincing analysis of repressed enzyme formation in the pathway leading to pyrimidine compounds has been carried out by R. A. Yates and A. B. Pardee (*J. Biol. Chem.* [in press] and personal communication).

[2] Induction and repression may respectively be brought about by inducers and repressers acting separately. When, however, an inducer and a represser of one and the same enzyme are present simultaneously, an antagonistic effect involving the two regulators may be expected to result. More generally, it is possible that inducer or represser action is antagonizable by substances that, in themselves, may or may not be regulators.

[3] S. Spiegelman and A. M. Campbell, in D. E. Green (ed.), *Currents in Biochemical Research, 1956* (New York: Interscience Publishers, Inc., 1956), p. 115.

[4] M. R. Pollock, in E. F. Gale and R. Davies (eds.), *Adaptation in Micro-organisms* (Cambridge: At the University Press, 1953), p. 150.

[5] C. E. Dalgliesh, *Science*, **125**, 271, 1957.

[6] F. Haurowitz, *Chemistry and Biology of Proteins* (New York: Academic Press, Inc., 1950).

[7] M. Cohn and J. Monod, in E. F. Gale and R. Davies (eds.), *Adaptation in Micro-organisms* (Cambridge: At the University Press, 1953), p. 132.

[8] Final shaping may include one or more of the following: (*a*) the folding or other intramolecular arrangement of a nascent template product; (*b*) the aggregation of products from the same or from different templates; (*c*) the combination of a template product with another type of substance; (*d*) the breaking of one or more bonds within a template product; and (*e*) the removal of a portion of a template product. The term "dynamic site" refers to that region of an enzyme which is primarily associated with the enzyme's characteristic catalytic action.

[9] J. Monod, *Growth*, **11**, 223, 1947.

[10] F. Lipmann, in D. E. Green (ed.), *Currents in Biochemical Research, 1956* (New York: Interscience Publishers, Inc., 1956), p. 241.

[11] Clearly, regulator action may depend either on both these effects or primarily on one or the other.

[12] J. Monod and M. Cohn, *Advances in Enzymol.*, **13**, 67, 1952.

[13] J. Monod, G. Cohen-Bazire, and M. Cohn, *Biochim. et Biophys. Acta*, **7**, 585, 1951. This paper includes an example of the specific inhibition of induced enzyme formation by an analogue of the inducer. Such inhibition would fall under the definition of enzyme repression given previously (Vogel, *op. cit.*).

[14] M. Cohn and A. Torriani, *Biochim. et Biophys. Acta*, **10**, 280, 1953. See also M. Cohn, Discussion of paper by Cohn and Monod, *op. cit.*, p. 149.

[15] J. Mandlestam, *Internatl. Rev. Cytol.*, **5**, 51, 1956.

[16] J. Yudkin, *Biol. Revs.*, **13**, 93, 1938.

[17] J. Mandlestam, *Biochem. J.*, **51**, 674, 1952.

[18] S. Spiegelman, *Cold Spring Harbor Symposia Quant. Biol.*, **11**, 256, 1946.

[19] J. Monod, in O. H. Gaebler (ed.), *Enzymes: Units of Biological Structure and Function* (New York: Academic Press, 1956), p. 7.

[20] The inducer would thus act as the master model or prototype of the (structurally complementary) dynamic site.

THE SITES OF ORTHOPHOSPHATE UPTAKE BY BARLEY ROOTS

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Kinetic analysis of measurements of steady-state uptake has established the extent to which the $HPO_4^{=}$ and $H_2PO_4^{-}$ ions contribute to the total uptake of orthophosphate by excised barley roots.[1] The binding compounds involved in the rate-limiting step of orthophosphate uptake are shown here to be components of the

respiratory chain. Further, the point where each phosphate species is coupled to the electron-transfer system is shown to be the site of energy conservation during oxidative phosphorylation by mammalian mitochondria.[2-4] The bearing of these results on theories of ion uptake is not developed. Discussion rather awaits results on the pathways of uptake of ions other than phosphate.

KINETICS OF PHOSPHATE UPTAKE

Uptake of ions by plant roots arises from an initial combination between an ion and a specific binding compound, as written in equation (1). Breakdown of this labile ion complex results in the uptake of ions into the system, as expressed by equation (2).

$$R + M_{\text{outside}} \underset{k_2}{\overset{k_1}{\rightleftarrows}} MR, \tag{1}$$

$$MR \underset{k_4}{\overset{k_3}{\rightleftarrows}} R' + M_{\text{inside}}, \tag{2}$$

where R represents a binding site or compound; M, the ion; MR, the labile intermediate; k, the rate constant for each reaction. The rate constants of association and dissociation, k_1 and k_2, of the intermediate, MR, are large with respect to the rate constant, k_3, of the breakdown of the intermediate; k_4 is negligible.

Uptake of the ion into the root may be described by the velocity, v, in the linear equation (3), when the concentration of the external ion is held constant and the concentration of R is constant during the period of absorption. Equation (3) is analogous to the steady-state expression describing the kinetics of enzyme reactions.[5, 6]

$$v = -\frac{v}{[M]} K_m + V_{\max}. \tag{3}$$

The maximum uptake at infinite ion concentration is $V_{\max}$, and K_m denotes the apparent dissociation constant of the intermediate ion complex, MR. When the steady-state uptake, v, by excised barley roots from single-salt solutions of alkali cations,[7] as well as halides,[8] is plotted against $v/[M]$, a straight line results over a wide range of concentrations. Thus a single first-order reaction is involved in the rate-limiting step of uptake for each of these monovalent ions, with the maximum uptake, $V_{\max}$, given directly by the ordinate intercept and the apparent K_m by the slope, according to equation (3). The steady-state uptake measurements of orthophosphate, when plotted in this manner, are curvilinear, because two first-order reactions are acting simultaneously, but independently on different substrates which are in a non-rate-limiting equilibrium.

Steady-state analysis of the measurements of orthophosphate uptake from solutions containing various concentrations of orthophosphate (10^{-6}–10^{-3} M) and hydrogen ion (pH 4–8) leads to the quantitative interpretation that excised barley roots absorb the ion species $H_2PO_4^-$ through one site and $HPO_4^=$ through another.[1] The system is described by the following reactions:

$$H_3PO_4 \rightleftarrows H^+ + H_2PO_4^- \rightleftarrows H^+ + HPO_4^= \rightleftarrows H^+ + PO_4^{\equiv}, \tag{4}$$

$$R_a^+ + H_2PO_4^- \rightleftarrows R_aH_2PO_4, \quad (5)$$

$$R_b^+ + HPO_4^= \rightleftarrows R_bHPO_4^-. \quad (6)$$

Further, the hydroxyl ion, OH^-, competitively inhibits the uptake of both ion species without affecting the concentration of either of the binding sites:

$$R_a^+ + OH^- \rightleftarrows R_aOH, \quad (7)$$

$$R_b^+ + OH^- \rightleftarrows R_bOH. \quad (8)$$

The concentration of all the intermediate ion complexes, expressed in equations (5)–(8), approximate equilibrium values, since their rate constants of association and discussion are large in comparison with the rate constant of breakdown, k_3. Thus the observed velocity of steady-state uptake, v, is directly proportional to the concentration of the ion-complex intermediates at all values of orthophosphate concentration. However, the total phosphate associated with the root, as measured in uptake studies, also includes the equilibrium value of the phosphate in combination with both sites. The observed uptake where these values contribute a significant amount to the total is denoted as v', as expressed in equation (9):

$$v' = \{[R_aH_2PO_4]k_{3a} + [R_bHPO_4^-]k_{3b}\}t + [R_aH_2PO_4] + [R_bHPO_4^-]. \quad (9)$$

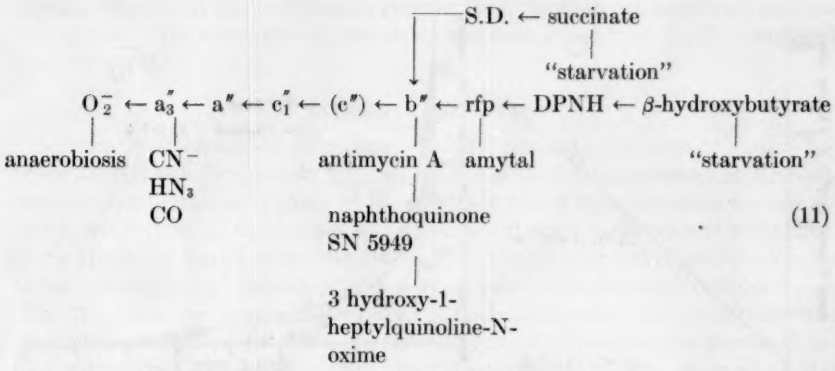
Sorption periods measured in seconds show a significant amount of phosphate in association with the roots because of the equilibrium value of the intermediate phosphate-site complexes (Fig. 1). Sixty-second absorption measurements must, therefore, be expressed by equation (9), where a plot of v' against $v'/[\Sigma P]$ (Fig. 2) results in a numerical value of the ordinate intercept which describes $V_{max\ a}$ and $V_{max\ b}$ for each reaction. The numerical values of $[R_aH_2PO_4]$ and $[R_bHPO_4^-]$ are equal to $[R_a]$ and $[R_b]$ at infinite substrate concentration ($v'/[\Sigma P] = 0$), since the sites exist completely in the combined form. Therefore, the maximum uptake for 1-minute absorption periods is expressed as follows:

$$V'_{max\ a} + V'_{max\ b} = \{[R_a]k_{3a} + [R_b]k_{3b}\}t + [R_a] + [R_b]. \quad (10)$$

Inspection of equation (9) shows that the phosphate in combination with the sites becomes negligible with respect to the total phosphate absorbed with longer periods of uptake, t. The 3-hour sorption periods used in previous studies[1] were of sufficient duration to express uptake solely as a velocity.

RELATIONSHIP OF PHOSPHATE UPTAKE TO RESPIRATION

The hypothesis for electron transport in the oxidative phosphorylation system has been extended by the accurate measurement and rigorous interpretations of the effects of substrates and inhibitors on the oxidation-reduction levels of the components of the respiratory chain.[9, 10] Certain inhibitors intercept electron transport at different points along the respiratory chain. These inhibitors cause those components on the oxygen side of the interaction to become more oxidized, while those on the substrate side become more reduced. Other types of inhibitors restrict or block electron flow and cause a difference in the steady-state oxidation-reduction level of the components of the respiratory chain. Each type of inhibitor has been studied with respect to the point of interruption of electron transport,[9] as expressed by the following scheme:



(11)

The sites at which energy-conservation reactions occur in isolated mammalian liver mitochondria[2-4] and in intact baker's yeast[4, 9] have been shown to be reduced cytochrome c, reduced cytochrome b, and DPNH.

A kinetic interpretation of the effect of similar respiratory chain inhibitors on the absorption of orthophosphate shows that reduced cytochrome b and DPNH are the components of the respiratory chain involved in the rate-limiting steps of orthophosphate uptake by excised barley roots.

The method for determining the components of the respiratory chain taking part in the rate-limiting steps of orthophosphate uptake is based on the following applications of first-order reaction kinetics:

a) A separation of the component reactions involved in both the presence and absence of an inhibitor, according to Figure 2, determines whether the effect is on one or both of the first-order reactions involved in orthophosphate uptake.

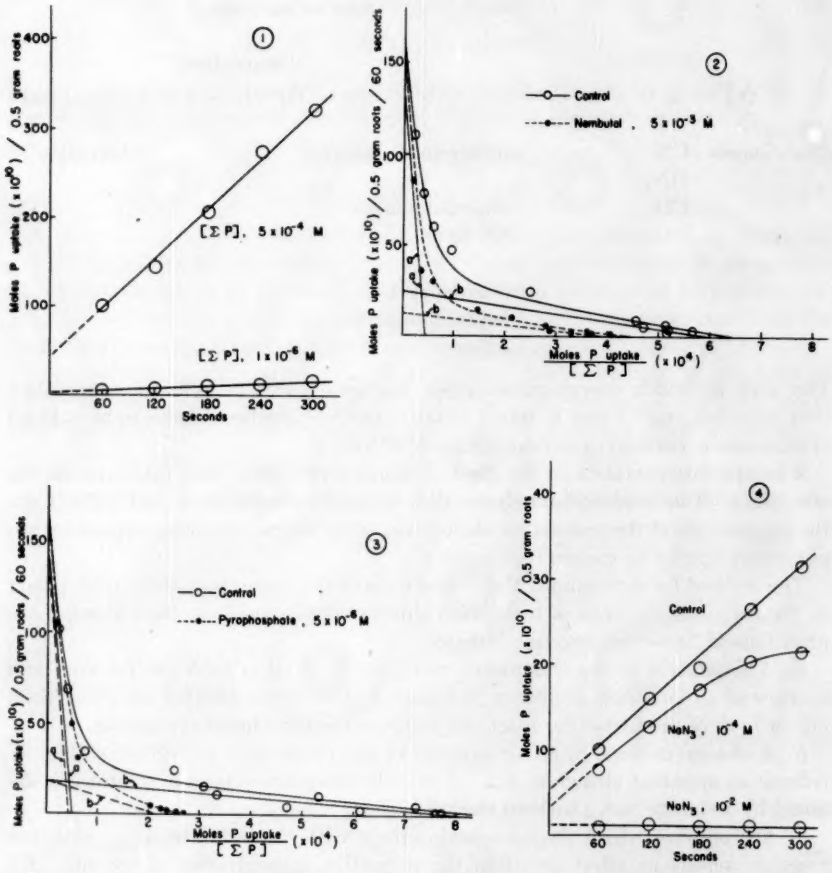
b) A change in slope of either reaction in the presence of an inhibitor (Fig. 3) reflects an apparent change in K_m. A strictly competitive type of inhibitor is denoted by a change in K_m without change in V'_{max}.

c) An inhibitor which changes the intercept with the ordinate, V'_{max}, of either reaction reflects an effect on either the respective concentration of the site, $[R]$, k_3, or both, according to equation (10).

d) Measurements of uptake with time permit a further separation of the effect of an inhibitor which changes the V_{max} of either reaction. When absorption is plotted against time in the presence of an inhibitor, a change in the slope of the resulting curve, with no change in the ordinate intercept, reflects an effect solely on k_3, according to equation (10). An effect on $[R]$ is reflected by both the slope and the intercept changing by the same ratio in the presence of an inhibitor.

EXPERIMENTAL METHODS

Excised roots from 6-day-old seedlings of *Hordeum vulgare*, variety Atlas 46, were used as the experimental material. The seedlings were grown essentially as described by Epstein and Hagen.[7] The seeds were soaked for 24 hours in continuously aerated demineralized water and then planted on a layer of cheesecloth supported by a stainless-steel screen suspended over a solution of 2×10^{-4} *M* $CaSO_4$. Roots were excised from the seedlings, which were grown for 5 days in a dark chamber maintained at 24° C.



FIGS. 1–4.—The uptake of orthophosphate by 0.5 gm. of excised barley roots. Fig. 1, from 1×10^{-6} and 5×10^{-4} *M* orthophosphate as a function of time. Fig. 2, as a function of orthophosphate uptake divided by total orthophosphate concentration with separation of the component reactions, in the presence and absence of nembutal. Fig. 3, as a function of orthophosphate uptake divided by total orthophosphate concentration with separation of the component reactions, in the presence and absence of pyrophosphate. Fig. 4, from 2×10^{-6} *M* orthophosphate in the presence and absence of azide.

The concentration of orthophosphate remained essentially constant over an absorption period of 5 minutes, using 25-ml. quantities of solution with each 0.5-gm. portion of excised roots. The roots, which were placed in test tubes, were rinsed three times with demineralized water. Single-salt solutions, at pH 4.0, of orthophosphate containing P^{32}, both in the absence and in the presence of various concentrations of substrate or inhibitor, were added to the roots at zero time. The pH value remained constant to within 0.1 unit during the absorption periods. At pH 4, the substrates and inhibitors were predominantly in the undissociated form, which has been shown to penetrate cells more readily than the charged ions.[11–13] At the end of the absorption period, the roots were rinsed four times with deminer-

alized water, which was sufficient to remove the phosphate not associated with active uptake. The absorption of phosphate was determined from the P^{32} associated with the roots.[1]

EXPERIMENTAL RESULTS

Location of Phosphate-binding Sites.—Amytal intercepts electron transport between DPNH and flavoprotein.[9] The presence of this inhibitor causes all the components in the respiratory chain on the substrate side of flavoprotein to become reduced, while those on the oxygen side become oxidized. A decrease of orthophosphate uptake by barley roots was observed on testing with either amytal or nembutal. Separation of the component reactions showed a decrease solely of $V'_{\max b}$ (Fig. 2). Both the slope and the intercept decreased by the same ratio when the phosphate uptake was measured as a function of time; therefore, the decreased uptake of the $HPO_4^=$ ion was due to a decrease solely of $[R_b]$. The action of amytal and nembutal establishes that the rate-limiting step of $HPO_4^=$ uptake could be either an oxidized component of the respiratory chain on the substrate side of flavoprotein or a reduced component on the oxygen side.

Paraphenylenediamine has been demonstrated to be a reductant of cytochrome c. All components in the respiratory chain toward the substrate side of cytochrome c become reduced in its presence. An increase in uptake occurred when the effect of this inhibitor was tested on orthophosphate uptake. Similar results were obtained with naphthoquinones, methylene blue, and 2,3-dimercaptopropanol. Separation of the component reactions showed an increase in $V'_{\max b}$, with no effect on $V'_{\max a}$. Measurement of the phosphate uptake as a function of time established that $[R_b]$ had been increased, since both the intercept and the slope increased by the same ratio. The deduction is that the rate-limiting step of $HPO_4^=$ uptake is coupled to reduced cytochrome b, since only changes in $[R_b]$ are involved both in the increase of $HPO_4^=$ uptake in the presence of this type of inhibitor and in the decrease of $HPO_4^=$ uptake in the presence of nembutal and amytal. Further, the uptake of $H_2PO_4^-$ is coupled to a reduced component of the electron-transfer system toward the substrate side of flavoprotein.

Addition of acetoacetate, the product of β-hydroxybutyrate oxidation, inhibited both $HPO_4^=$ and $H_2PO_4^-$ uptake. The inhibition was a function of a decrease in $V'_{\max b}$ and $V'_{\max a}$. The inhibition was further resolved by time studies to show that a decrease solely in $[R_a]$ and $[R_b]$ had occurred. Thus $H_2PO_4^-$ uptake is coupled to a reduced component of the respiratory chain which lies between the substrate and flavoprotein: the DPNH.

Systems in the steady state of aerobic metabolism generally maintain cytochrome b in a considerably less reduced state than DPNH, which is largely reduced. A measurable effect on $HPO_4^=$ uptake could occur with a negligible change in $H_2PO_4^-$ uptake in the presence of inhibitors which cause both cytochrome b and DPNH to become more reduced. This is the case, apparently, in the nonmeasurable effect of *p*-phenylenediamine, nembutal, and amytal on the DPNH which is coupled to $H_2PO_4^-$.

Site Concentrations.—Chance and Williams[14] have shown that the concentration of DPNH exceeds many fold that of cytochrome b in intact mammalian mitochondria. The relative concentrations of the two sites involved in the rate-limiting

step of orthophosphate uptake by barley roots may be approximated without reference to the chemical identity of the sites. The ratio of the concentrations provides a criterion for corroboration of cytochrome b and DPNH as the two sites of uptake. Most of the total phosphate associated with the roots from solutions of 1×10^{-6} M orthophosphate (where $v'_{a+b}/[\Sigma P] = 5 \times 10^{-4}$) is contributed by the $HPO_4^{=}$ ion (Fig. 2). Thus an approximation of $[R_bHPO_4^{-}]$ can be determined from the value of the intercept at zero time obtained from extrapolation of uptake measurements with time (Fig. 1). A value of k_{3b} can be calculated by substitution of a value of v_o' and $[R_bHPO_4^{-}]$ at 1×10^{-6} $M[\Sigma P]$, obtained from Figure 1, into the expression for $HPO_4^{=}$ uptake, equation (12):

$$v_b' = [R_bHPO_4^{-}]k_{3b}t + [R_bHPO_4^{-}]. \quad (12)$$

Solution of the equation gave a value for k_{3b} of 2.3×10^{-2} moles P/mole $R_b \times$ sec. The steady-state uptake of $HPO_4^{=}$ is directly proportional to the concentration of the $R_bHPO_4^{=}$ at all values of orthophosphate concentration. Thus a value for $[R_b]$ of 11.4×10^{-10} moles R_b/0.5 gm roots is obtained when the value for k_{3b} is substituted in equation (13), which expresses the maximum absorption where $[R_b]$ is completely saturated with $HPO_4^{=}$; the $[R_b]$ is equal to $[R_bHPO_4^{-}]$:

$$V'_{\max\ b} = [R_b]k_{3b}\,t + [R_b]. \quad (13)$$

Essentially total saturation of R_b is obtained at 5×10^{-4} M orthophosphate concentrations (where $V'_{a+b}/[\Sigma P] = 0.2 \times 10^{-4}$) (Fig. 2) and $[R_bHPO_4^{-}]$ is approximately equal to $[R_b]$. The value of $[R_aH_2PO_4]$ is obtained by subtraction of $[R_b]$, since the intercept of the time curve for concentrations of orthophosphate of 5×10^{-4} M is a measure of phosphate in combination with both sites (Fig. 1). Total uptake at 5×10^{-4} M $[\Sigma P]$ may therefore be expressed as

$$v'_{a+b} = \{[R_aH_2PO_4]k_{3a} + [R_b]k_{3b}\}t + [R_aH_2PO_4] + [R_b], \quad (14)$$

where numerical values of $[R_aH_2PO_4]$, $[R_b]$, k_{3b} are known approximately and the value of v'_{a+b} is obtained from Figure 1. Solution of equation (14) gave a value of k_{3a} of 1.4×10^{-6} moles P/mole $R_a \times$ sec. Substitution of this value into an expression for $V'_{\max\ a}$ results in a value for $[R_a]$ of 78×10^{-10} moles R_a/0.5 gm roots. The relative concentration of R_b to R_a must be at least 1:7, a ratio which is of the same magnitude as the ratio of cytochrome b to DPNH in mammalian mitochrondria.

Actions of Competitive Inhibitors.—Adenosine diphosphate and pyrophosphate similarly inhibited orthophosphate uptake by barley roots. In the presence of a 5×10^{-6} M concentration of either inhibitor, a separation of the component reactions showed a change in K_{mb} with no change in $V'_{\max\ b}$ (Fig. 3), with no measurable effect on the $H_2PO_4^{-}$ uptake at this concentration. Within the range of inhibitor concentrations from 1×10^{-4} to 1×10^{-1} M, ADP and pyrophosphate decreased the uptake of both $HPO_4^{=}$ and $H_2PO_4^{-}$. The inhibition was shown to be a function solely of a change in apparent K_{ma} and K_{mb}. The inhibition of $H_2PO_4^{=}$ uptake became apparent only at the high concentrations of the inhibitor, because of a lesser affinity for the $H_2PO_4^{-}$ site than for the $HPO_4^{=}$ site. The effect of either ADP or pyrophosphate was competitive to the uptake of both $H_2PO_4^{-}$ and $HPO_4^{=}$, since the inhibition was abolished by increasing concentrations of orthophosphate.

Several schemes describing oxidative phosphorylation implicate an acceptor which forms an inorganic phosphate–ADP complex. Since R_a and R_b exist completely in the forms $R_aH_2PO_4$ and $R_bHPO_4^-$ at infinite orthophosphate concentrations, additions of ADP would be expected to increase the maximum uptake, $V'_{\max\ a+b}$, if such a complex were essential to uptake. However, $V'_{\max\ a}$ and $V'_{\max\ b}$ are unchanged by the additions of ADP. Either the endogenous ADP differs from the ADP in solution, or a phosphate-ADP complex is not required in the rate-limiting step of orthophosphate uptake by barley roots.

The competitive inhibition by hydroxyl ion,[1] DNP,[15] ADP, and pyrophosphate implies that these competitive inhibitors combine with the same sites involved in the rate-limiting steps of orthophosphate uptake: the reduced cytochrome b and DPNH. The relatively high affinity of the hydroxyl ion[1] suggests that the sites of phosphorylation may exist primarily in combination with the hydroxyl ion. Competitive inhibition of orthophosphate uptake implies a direct reaction of the orthophosphate species and the competitive inhibitors with the sites involved. First-order reaction kinetics cannot exclude the possibility that the actual competition occurs at some intermediates which are coupled to cytochrome b and DPNH.

Inhibitors Which Block Electron Flow.—Azide is believed to block electron flow by combining with the oxidized form of the terminal oxidase, cytochrome a_3, which results in the components of the respiratory chain becoming more reduced.[3] The presence of a low concentration of azide (10^{-6}–10^{-5} M) caused a small increase in uptake from 10^{-6} M orthophosphate. The increased phosphate uptake was a function primarily of an increased $[R_b]$, with no measurable change in the value of k_{3b}. A decrease in the apparent rate of breakdown of the phosphate-site complexes, k_{3a+b}, is obligatory upon an electron restriction, since oxidative phosphorylation is a direct function of electron flow. The apparent absence of a change in the value of k_{3b} is in accordance with Chance's observation that large changes in the oxidation-reduction levels of components involved in electron transfer are observed with only a small inhibition of respiration over a considerable range of azide concentrations. With greater azide concentrations (10^{-4} M), the rate of phosphate uptake decreased with increasing times of sorption (Fig. 4). This deviation from steady-state uptake was peculiar to this type of inhibitor. Inspection of the initial slope of the time curve, where steady-state uptake was approximated (Fig. 4), showed that a decrease in $[\Sigma R]$ had occurred. Further, a separation of the component reactions after 1-minute sorption periods ascribed the inhibition to a decrease of $[R_b]$. Increasing concentrations of orthophosphate did not overcome the inhibition. Thus, to be consistent with the previous interpretations, an oxidation of cytochrome b, R_b, must be assumed at these concentrations of azide. In the presence of even greater concentrations of azide (10^{-2} M), a drastic decrease in the apparent k_{3a+b} was evident, as well as an even further decrease in $[\Sigma R]$. Separation of the component reactions showed that the phosphorylations coupled to both R_a and R_b are decreased. Increasing orthophosphate concentrations did not overcome the inhibition in either case, and an oxidation of both cytochrome b and DPNH must have occurred. The contribution of $HPO_4^=$ uptake is small in comparison with total uptake in the presence of 5×10^{-4} M orthophosphate. At this phosphate level, uptake is inhibited over the whole range of azide concentrations, with a significant decrease in both $[R_a]$ and k_{3a}.

The substrate-inhibitor method for the examination of a respiratory chain is based on the premise that components of the respiratory chain on the oxygen side of the point of action of an inhibitor become more oxidized and those on the substrate side more reduced. The increased uptake of orthophosphate at low azide and phosphate concentrations is in accordance with the reduction of the components of the respiratory chain induced by an azide block of electron flow at cytochrome a_3. However, higher concentrations of azide inhibit uptake by decreasing both the k_{3a+b} and $[R_{a+b}]$. A secondary site of action of azide with a respiratory component toward the substrate side of DPNH must be assumed on the basis of the substrate-inhibitor method. Further, the deviation from steady-state uptake with time suggests that azide combines with a state of the respiratory component which is a product arising from electron transfer. The conclusion is that azide combines with the oxidized form, DPN, as well as with cytochrome a_3, on the basis that the current hypothesis adequately describes the components of the respiratory chain involved in oxidative phosphorylation (eq. [11]).

The recent finding that cyanide combines with the oxidized form, DPN,[16] suggested a study of the effect of cyanide on orthophosphate uptake. The characteristics of the cyanide inhibition were identical with those of azide, although slightly greater effective concentrations of cyanide were required to effect the same degree of inhibition as azide.

Action of Substrates.—The respiratory chain responds differentially to the type of substrate which is dehydrogenated. The respiration rates of intact mammalian mitochondria, for instance, have been shown to be about three times larger with succinate as a substrate than with β-hydroxybutyrate.[17] Further, a greater reduction of components of the respiratory chain occurred with the greater rate of electron flow effected by succinate.[18]

Mitochondrial preparations, after starvation periods of relatively short duration, essentially are depleted of endogenous substrates. However, excised barley roots are not amenable to a quantitative depletion of endogenous substrates, and addition of a high concentration of substrate is required for assurance that the measured response is a function primarily of the prevailing substrate.

Succinate addition to an effective concentration of 1×10^{-1} M decreased the uptake of orthophosphate by excised barley roots. Separation of the component reactions showed the inhibition to be essentially a function of a decrease in $V_{\max\ a}$. Measurement of phosphate uptake as a function of time showed that the slope decreased to a lesser extent than the intercept (Fig. 5). Thus the effects of a high concentration of succinate on phosphate uptake were resolved as a decrease in $[R_a]$ and an increase in the apparent k_{3a+b}. The prevailing concentration of succinate severely restricts electron flow through the competing pathway of β-hydroxybutyrate. The decrease in $[R_a]$, therefore, results from the DPNH becoming more oxidized with the slower rate of electron flow through the β-hydroxybutyrate pathway.

A significantly larger decrease in uptake was effected by a 1×10^{-1} M concentration of β-hydroxybutyrate. An analysis of the effect, identical with that used for succinate, resulted in the conclusion that β-hydroxybutyrate had decreased significantly both $[R_b]$ and the apparent k_{3a+b}. A severe restriction of electron flow through the succinate pathway is effected by the competing pathway of β-hydroxy

butyrate. This effect alone would not necessarily decrease $[R_b]$, since both cytochrome b and DPNH are involved in the pathway of the reducing reaction. However, the decrease in k_{3a+b} showed that a slower rate of electron flow was effected by this substrate than by succinate. The slower electron flux results in cytochrome b becoming more oxidized.

These experimental observations are in qualitative agreement with the current hypothesis of oxidative phosphorylation in the electron transport system.[9] Inspection of the chemical representation of the respiratory chain (eq. [11]) shows that the electrons from both the succinate and the β-hydroxybutyrate pathways of reducing reactions converge on cytochrome b. The dehydrogenation of a substrate such as β-hydroxybutyrate, which requires DPN in the pathways of reducing reactions, results in the uptake of both the $H_2PO_4^-$ and $HPO_4^=$ ions which are coupled to DPNH and reduced cytochrome b, respectively. The values of k_{3a} and k_{3b} must be equal, since the rate of breakdown of both $R_aH_2PO_4$ and $R_bHPO_4^=$ is a direct function of electron flow, and the breakdown of both occurs with the transfer of an electron pair. The magnitude of their numerical value is a measure of the rate of electron flow effected by the β-hydroxybutyrate pathway.

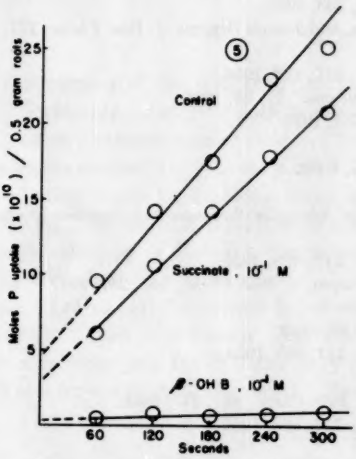


FIG. 5.—The uptake of orthophosphate from 0.5 gm. of excised barley roots from 1×10^{-6} *M* orthophosphate in the presence and absence of succinate or β-hydroxybutyrate.

The succinate pathway does not involve DPNH but is linked to cytochrome b. In this instance, however, the numerical value of k_{3b} is a measure of the rate of electron flow effected by the succinate pathway. The value of k_{3a+b} was shown to be increased by succinate and decreased by β-hydroxybutyrate. A greater value of k_{3b} must have occurred in the presence of succinate than with β-hydroxybutyrate, since k_{3a} is a function of β-hydroxybutyrate dehydrogenation. Therefore, a more concise description must be accorded k_{3b}—namely, $k_{3b\ \text{succinate}}$ and $k_{3b\ \beta\text{-OHB}}$. The apparent k_{3b} of control roots, utilizing an endogenous level of substrate, is a numerical value dependent on the Michaelis affinities, concentration, and ratio of the substrates.

Approximate values for $k_{3b\ \text{succinate}}$, $k_{3b\ \beta\text{-OHB}}$, and k_{3a} may be calculated on the supposition that a high concentration of an added substrate effects a single pathway of electron flow. Values of $[R_b]$ and v_b', obtained from the succinate curve of Figure 5, are substituted in equation (12), which expresses the uptake of the $HPO_4^=$ ion. Solution of the equation resulted in a value of 2.1×10^{-2} moles P/mole $R_b \times$ sec. Similar treatment of the measurements of uptake from 1×10^{-1} *M* β-hydroxybutyrate resulted in a value of 8.3×10^{-3} moles P/mole $R \times$ sec. This value expresses either k_{3a} or $k_{3b\ \beta\text{-OHB}}$, since both are equal in the β-hydroxybutyrate pathway.

The 2.5 times larger uptake of the $HPO_4^=$ ion with succinate than with β-hydroxybutyrate as a substrate is in close agreement with the 3 times larger respiration rate

shown with the same substrates by Chance and Williams for mammalian mitochondria.

Also, the value of 2.1×10^{-2} moles P/mole $R \times$ sec for $k_{3b\ \text{succinate}}$ is in essential agreement with the value of 2.3×10^{-2} moles P/mole $R \times$ sec for the k_{3a} calculated from kinetic analysis of uptake measurements of control roots utilizing an endogenous level of substrate. However, the value of 8.3×10^{-3} moles P/mole $R \times$ sec for either k_{3a} or $k_{3b\ \beta\text{-OHB}}$ is much lower than the value for k_{3a} of 1.4×10^{-2} moles P/mole $R \times$ sec which was calculated from uptake measurements of control roots. A study of the effect on orthophosphate uptake of numerous substrates which require DPN in the pathways of reducing reactions has resulted in the conclusion that the k_{3a} of glutamate corresponds most closely to the k_{3a} calculated from uptake measurements of excised barley roots utilizing an endogenous level of substrate.

CONCLUSIONS

Orthophosphate uptake by barley roots is coupled to sites identical with those for oxidative phosphorylation during electron transport in mammalian mitochondria.

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*THE NORM FORM OF A RATIONAL DIVISION ALGEBRA**

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Let $\mathfrak{D}$ be a finite-dimensional associative division algebra over the field $\mathfrak{R}$ of all rational numbers. Then the center $\mathfrak{F}$ of $\mathfrak{D}$ is an algebraic extension of finite degree over $\mathfrak{R}$, and $\mathfrak{D}$ is a cyclic algebra of degree n and dimension n^2 over $\mathfrak{F}$. We may then write $\mathfrak{D} = (\mathfrak{Z}, S, \gamma)$, where $\mathfrak{Z}$ is a cyclic field of degree n over $\mathfrak{F}$, γ is a nonzero

element of $\mathfrak{F}$, and S generates the cyclic automorphism group of $\mathfrak{Z}$ over $\mathfrak{F}$. The general element x of $\mathfrak{D}$ now has the form $x = x_0 + x_1y + \cdots + x_{n-1}y^{n-1}$, where the x_i are in $\mathfrak{Z}$, and y in $\mathfrak{D}$ has the property $y^n = \gamma$, $yz = (zS)y$.

The algebra $\mathfrak{D}$ now has an isomorphic representation as an algebra of n-rowed square matrices with elements in $\mathfrak{Z}$. It is the mapping $x \to X = X_0 + X_1Y + \cdots + X_{n-1}Y^{n-1}$, where $X_i = \text{diag}\{x_i, x_iS, \ldots, x_iS^{n-1}\}$ and

$$Y = \begin{bmatrix} 0 & 1 & 0 \ldots 0 & 0 \\ 0 & 0 & 1 \ldots 0 & 0 \\ . & . & . \ldots . & . \\ 0 & 0 & 0 \ldots 0 & 1 \\ \gamma & 0 & 0 \ldots 0 & 0 \end{bmatrix}.$$

Then we define the *norm form* of $\mathfrak{D}$ to be the determinant

$$\Delta(x) = |X|$$

of the representation matrix. It is a homogeneous polynomial of degree n in the n^2 co-ordinates *in* $\mathfrak{F}$ of the general element x of $\mathfrak{D}$, and it *permits composition*, that is

$$\Delta(xy) = \Delta(x) \cdot \Delta(y).$$

The purpose of this note is to provide a proof of the following result.

THEOREM. *Let $\mathfrak{D}$ be a division algebra of odd prime degree over its center $\mathfrak{F}$, where $\mathfrak{F}$ is an algebraic number field. Then the norm form of $\mathfrak{D}$ is universal, that is, every nonzero element δ of $\mathfrak{F}$ is the norm $\delta = \Delta(x)$ of an element x in $\mathfrak{D}$.*

Let us begin by recalling some of the properties of rational division algebras.[1] We let $\mathfrak{J}$ be the ring of all integers of $\mathfrak{F}$, so that every prime ideal $\mathfrak{P}$ of $\mathfrak{J}$ determines a *valuation* $\phi_\mathfrak{P}$ of $\mathfrak{F}$, and a *completion* $\mathfrak{F}_\mathfrak{P}$ of $\mathfrak{F}$. The scalar extension $\mathfrak{D}_\mathfrak{P}$ of $\mathfrak{D}$ by $\mathfrak{F}_\mathfrak{P}$ will then be a total matric algebra for all but a finite number[2] of valuations $\phi_\mathfrak{P}$. Let us call the primes $\mathfrak{P}$ for which $\mathfrak{D}_\mathfrak{P}$ is a division algebra the *critical primes* for $\mathfrak{D}$. Then it is known[3] that a field $\mathfrak{K}$, of the given odd prime degree n over $\mathfrak{F}$, is a *splitting field* for $\mathfrak{D}$ if and only if the degree $n_\mathfrak{P}$ of each composite $(\mathfrak{K}, \mathfrak{F}_\mathfrak{P})$ over $\mathfrak{F}$ is n for every critical prime. Thus, if $\mathfrak{K} = \mathfrak{F}[\xi]$, where ξ is a root of

$$f(\omega) = \omega^n + \alpha_1\omega^{n-1} + \cdots + \alpha_n,$$

with α_i in $\mathfrak{F}$, and $f(\omega)$ is irreducible in $\mathfrak{F}_\mathfrak{P}$ for every critical prime $\mathfrak{P}$, it will follow that $\mathfrak{K}$ splits $\mathfrak{D}$. Then $\mathfrak{K}$ will be isomorphic to a subfield $\mathfrak{F}[x]$ of $\mathfrak{D}$, $f(x) = 0$, $\alpha_n = (-1)^n \Delta(x)$. It should now be clear that our theorem is equivalent to the property that *if α_n is any nonzero element of $\mathfrak{F}$, there exist elements $\alpha_1, \cdots, \alpha_{n-1}$ in $\mathfrak{F}$ such that $f(\omega)$ is irreducible in $\mathfrak{F}_\mathfrak{P}$ for any prescribed finite set of prime ideals $\mathfrak{P}$ of $\mathfrak{F}$.* However, it will be convenient for us to remain closer to the theory of algebras than this remark might suggest.

There is no loss of generality if we assume that α_n is an integer of $\mathfrak{F}$, since $\alpha_n = -\Delta(x)$ if and only if $\lambda^n\alpha_n = -\Delta(\lambda x)$. We can clearly select λ so that $\lambda^n\alpha_n$ is integral. We shall also select the elements $\alpha_1, \cdots, \alpha_{n-1}$ to be algebraic integers, so that any root ξ of $f(\omega) = 0$ is always integral. Every element of $\mathfrak{F}_\mathfrak{P}$ has the form $\alpha = \pi^\lambda\mu$, where π can be taken to be any element of $\mathfrak{P}$ not in $\mathfrak{P}^2$, and μ is a unit

of the ring $\mathfrak{J}_\mathfrak{P}$ of all integers of $\mathfrak{F}_\mathfrak{P}$. Then $\lambda \geq 0$ for every element of $\mathfrak{J}_\mathfrak{P}$. We take $\mathfrak{P}$ to be a critical prime for $\mathfrak{D}$ and write

$$\alpha_n = \pi^\lambda \beta_n, \qquad \lambda = nk + t \qquad (0 \leq t < n),$$

where β_n is a unit of $\mathfrak{J}_\mathfrak{P}$. If $t > 0$, we select the α_t so that

$$\alpha_t \equiv 0 \pmod{\mathfrak{P}^{nk+t}}. \tag{1}$$

Then $\alpha_t = \pi^{nk+t}\beta_t$ for integers β_t in $\mathfrak{J}_\mathfrak{P}$, and $f(\omega) = \omega^n + \pi^{nk}(\beta_1\pi\omega^{n-1} + \beta_2\pi^2\omega^{n-2} + \cdots + \beta_{n-1}\pi^{n-1}\omega + \pi^t\beta_n)$. As we have said, $f(\omega)$ has leading coefficient 1 and other coefficients in $\mathfrak{J}_\mathfrak{P}$, and so every root of $f(\omega)$ is integral over $\mathfrak{J}_\mathfrak{P}$. But then $f(\omega)$ of prime degree is reducible if and only if it has a root ρ in $\mathfrak{J}_\mathfrak{P}$, and we may write $\rho = \pi^\nu\sigma$ for a unit σ of $\mathfrak{J}_\mathfrak{P}$. Clearly π^{nk+1} divides ρ^n, and so $\nu = k + s$, where we must have $s > 0$. Then π^n is a factor of ρ^n as well as of $\beta_t\pi^t\rho^{n-1}$, and so π^n divides $\pi^t\beta_n$, which is impossible. Thus the congruences (1) make $f(\omega)$ irreducible in the field $\mathfrak{F}_\mathfrak{P}$ for every critical prime divisor $\mathfrak{P}$ of α_n such that the exact power of $\mathfrak{P}$ dividing α_n is $\mathfrak{P}^{kn+t}$, where $0 < t < n$.

There remains the case where $t = 0$, so that $\alpha_n = \pi^{kn}\beta_n$, with β_n a unit of $\mathfrak{J}_\mathfrak{P}$. We assume that

$$\alpha_t \equiv \pi^{nk-n+t}\gamma_t \pmod{\mathfrak{P}^{nk-n+t+1}}, \tag{2}$$

where the elements $\gamma_t \not\equiv 0 \pmod{\mathfrak{P}}$ will be selected later. Then $\alpha_t = \pi^{nk-t+t}\beta_t$, where β_t is a unit of $\mathfrak{J}_\mathfrak{P}$, and may be determined modulo π. But then $f(\omega) = \omega^n + \beta_1\pi^{nk-(n-1)}\omega^{n-1} + \cdots + \beta_{n-1}\pi^{nk-1}\omega + \pi^{nk}\beta_n$ has a root in $\mathfrak{F}_\mathfrak{P}$ if and only if $\pi^{-nk}f(\omega\pi^k) = g(\omega) = \omega^n + \beta_1\omega^{n-1} + \cdots + \beta_{n-1}\omega + \beta_n$ has a root in $F_\mathfrak{P}$. It is known[4] that the algebra $\mathfrak{D}_\mathfrak{P} = (\mathfrak{W}, T, \pi^e)$, where $\mathfrak{W}$ is a cyclic unramified field over $\mathfrak{F}_\mathfrak{P}$ with generating automorphism T, $\mathfrak{W} = \mathfrak{F}_\mathfrak{P}(h)$ for a root of unity h. The residue-class field $\mathfrak{H}_\mathfrak{P} = \mathfrak{J}_\mathfrak{P} - (\pi)$ is a finite field of q elements, and $\mathfrak{H}_\mathfrak{P}(h)$ has degree n over $\mathfrak{H}_\mathfrak{P}$, h is a primitive $(q^n - 1)$st root of unity, $hT = h^q$. Every unit of $\mathfrak{J}_\mathfrak{P}$ is known[5] to be a norm in $\mathfrak{W}$, and we observe that, if

$$a = \frac{hT}{h} = h^{q-1},$$

then the norm of a as an element of $\mathfrak{W}$ is 1. However, a is never in $\mathfrak{F}_\mathfrak{P}$, since otherwise the residue class of a has period $q - 1$, $h^{(q-1)(q-1)} \equiv 1 \pmod{\pi}$, whereas the period of h is $q^n - 1 > (q-1)^2$, since $q^{n-1} + \cdots + q + 1 > q - 1$ when $q \geq 2$. But if $\beta_n = -N(b)$ for b in $\mathfrak{W}$, and b is in $F_\mathfrak{P}$, then $\beta_n = -N(ab)$, where ab is not in $\mathfrak{F}_\mathfrak{P}$. It follows that $\beta_n = -N(b)$, where b *generates* the cyclic field $\mathfrak{W}$ of degree n over $\mathfrak{F}_\mathfrak{P}$. Then b is a root of $g_0(\omega) = \omega^n + \sigma_1\omega^{n-1} + \cdots + \sigma_{n-1}\omega + \beta_n$, and we can take

$$\beta_t \equiv \sigma_t \pmod{\mathfrak{P}}. \tag{3}$$

Since $\mathfrak{W}$ is unramified, the polynomial $[g_0(\omega)] = \omega^n + [\sigma_1]\omega^{n-1} + \cdots + [\sigma_{n-1}]\omega + [\beta_n]$, over the residue-class ring $\mathfrak{H}_\mathfrak{P}$, is irreducible. Then (3) implies that $g(\omega)$ is irreducible, for a reducible polynomial cannot have an irreducible residue class. Thus the selection (3) makes $f(\omega)$ irreducible in the cases of critical primes $\mathfrak{P}$ with α_n exactly divisible by $\mathfrak{P}^{nk}$. Since we have a finite number of congruences to be satisfied by the α_t, we can select them so that $f(\omega)$ is irreducible, as desired.

This completes our proof in the case where $\mathfrak{F}$ is an extension of finite degree over $\mathfrak{R}$. The result is true, however, even when $\mathfrak{F}$ is infinite-dimensional over $\mathfrak{R}$. For $\mathfrak{D}$ has a basis $u_1 = 1, u_2, \cdots, u_m$ over $\mathfrak{F}$ and a multiplication table $u_i u_j = \gamma_{ijk} u_k$ for algebraic numbers γ_{ijk}, where $m = n^2$. If $\delta \neq 0$ is in $\mathfrak{F}$, we let $\mathfrak{F}_0$ be the field obtained by adjoining the γ_{ijk} and δ to $\mathfrak{R}$. Then $\mathfrak{D} = (\mathfrak{D}_0)_{\mathfrak{F}}$, where $\mathfrak{D}_0 = u_1\mathfrak{F}_0 + \cdots + u_m\mathfrak{F}_0$ is a central division algebra of degree n over $\mathfrak{F}_0$. The field $\mathfrak{F}_0$ is a finite algebraic extension of $\mathfrak{R}$, and so $\delta = \Delta(x)$, where x is in D_0. But $\Delta(x)$ is the norm form of $\mathfrak{D}$, and our result is proved.

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[1] An exposition of this theory is given as chap. IX of the author's *Structure of Algebras*.

[2] See Theorem 9.30.

[3] This is a consequence of Theorems 9.31 and 9.23.

[4] See Theorem 9.21.

[5] See Theorem 9.19.

THE RANDOM FUNCTIONS OF COSMIC-RAY CASCADES

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1. *Introduction.*—We consider the electron-photon cascades of cosmic radiation in the formulation of Bhabha and Heitler[1] and of Carlson and Oppenheimer.[2] We make the simplifying assumptions (used in some of the physical literature) appropriate to the so-called "complete screening" case.[3] Hence the asymptotic results given below cannot be strictly true, since our assumptions hold only over limited ranges of the parameters. Presumably, detailed analytical study of more complicated models would be necessary to determine whether the results have some qualitative validity.

A photon of positive energy ϵ, moving through homogeneous material, has probability $\lambda\, dt + o(dt)$ (henceforth we shall omit the o-terms in such expressions) of being transformed in the thickness interval $(t, t + dt)$ into two electrons, positive and negative, which receive respective energies ϵu and $\epsilon(1 - u)$, $0 < u < 1$, with probability density $q(u)$. We assume that q has a bounded derivative for $0 \leq u \leq 1$ and is symmetric about $1/2$. An electron loses (by "collision" or "ionization") the deterministic amount of energy βt in any interval of length t, provided that the energy is not thereby reduced below 0; β and λ are constants independent of t and of the energy. Also, an electron radiates photons ("Bremsstrahlung"): the probability that an electron of energy ϵ emits a photon of energy between ϵu and $\epsilon(u + du)$ in the interval dt is $k(u)\, du\, dt$, the energy which goes to the photon being subtracted from that of the electron. The usual simplifying assumptions are covered if we take

$$k(u) = \frac{\mu}{u} + k_0(u), \qquad \left|\frac{dk_0}{du}\right| \leq c(1 - u)^{-\delta}, \qquad 0 \leq u < 1, \tag{1.1}$$

where c, μ are positive constants and $b < 2$. Since $\int_0^1 k(u)\,du = \infty$ (infinite cross-section), with probability 1 infinitely many photons are produced by an electron in each t-interval in which it has positive energy.

2. *The Straggling*[4] *and Generating Functions When* $\beta = 0$.—Let $\epsilon_0(t)$ be the energy at t of an electron which had initially energy 1, in the case $\beta = 0$. Putting $X_0(t) = -\log \epsilon_0(t)$, it can be seen that $X_0(t)$ has for $t > 0$ a distribution of the infinitely divisible type with a probability density $h_t(x)$ whose characteristic function is, for $x > 0$,

$$\int_0^\infty h_t(x)e^{irx}\,dx = \exp\left\{t\int_0^\infty (e^{iru} - 1)k(1 - e^{-u})e^{-u}\,du\right\}. \tag{2.1}$$

The case $k(u) = -\mu/\log(1 - u)$, where $h_t(x)$ is then $x^{\mu t-1}e^{-x}/\Gamma(\mu t)$, was given by Bethe and Heitler.[5]

Let $N(\epsilon, t)$ be the total number of electrons at t whose energies are greater than ϵ, and let $f_1(s, \epsilon, t)$ and $f_2(s, \epsilon, t)$ be the generating functions, $\sum_r P(N(\epsilon, t) = r)s^r$, if initially (i.e., at $t = 0$) there is, respectively, one photon or one electron of energy 1. To derive equations for f_1 and f_2, the usual method of regeneration points must be modified, since there is no first photon emission. Since differential equations for f_1 and f_2 have not previously been justified in the present case of infinite cross-sections, we mention that differentiability in ϵ and t of f_1 and f_2 can be established by direct consideration of the random process, and we get equation (2.3) below; the more simply derived companion equation (2.2) is also given:

$$\frac{\partial f_1(s, \epsilon, t)}{\partial t} = -\lambda f_1(s, \epsilon, t) + \lambda\int_0^1 f_2\left(s, \frac{\epsilon}{u}, t\right) f_2\left(s, \frac{\epsilon}{1 - u}, t\right) q(u)\,du, \tag{2.2}$$

$$\frac{\partial f_2(s, \epsilon, t)}{\partial t} = \int_0^1 \left\{f_1\left(s, \frac{\epsilon}{u}, t\right) f_2\left(s, \frac{\epsilon}{1 - u}, t\right) - f_2(s, \epsilon, t)\right\} k(u)\,du, \tag{2.3}$$

with $f_1(s, \epsilon, 0) = f_1(s, 1, t) = f_2(s, 1, t) = 1$, $t > 0$, and $f_2(s, \epsilon, 0) = s$ for $\epsilon < 1$. Uniqueness of the solution among a wide class of functions follows from the fact that the factorial moments (derivatives of the f_i with respect to s at $s = 1$) have Mellin transforms on ϵ which satisfy linear differential equations.

In the case of a finite total cross-section ($\int_0^1 k(u)\,du < \infty$), integral equations were given for the characteristic functional of $N(\epsilon, t)$ by Bartlett and Kendall[6] and for the generating functions f_1 and f_2 by Jánossy[7] and others. Integral equations can be obtained in the present case using a method similar to the one employed by Moyal[8] to get his equation (2.22).

3. *The Expectation Process When* $\beta = 0$.—Define a vector Markov process $(I(t), \zeta(t))$, $t \geq 0$, with $I = 1$ or 2 and $\zeta > 0$, by the following scheme (let $K = \int_0^1 uk(u)\,du$). $I(t)$ is itself a two-state temporally homogeneous Markov process (we can consider t as a "time" rather than a "thickness" parameter) with respective probabilities $\lambda\,dt$ and $K\,dt$ for the transitions $1 \to 2$ and $2 \to 1$ in dt. Whenever the transition $1 \to 2$ occurs, $\zeta(t)$ is multiplied by u, $0 < u < 1$, with probability density $2uq(u)$, and whenever $2 \to 1$ occurs, $\zeta(t)$ is multiplied by u with density $uk(u)/K$;

furthermore, if $I(t) \equiv 2$ for $t_1 \leq t \leq t_2$, then $\zeta(t)/\zeta(t_1)$ decreases, in the interval $t_1 \leq t \leq t_2$, in the manner of the straggling process of Section 2, except that the function $k(u)$ is replaced by $(1 - u)k(u)$; $\zeta(t)$ remains constant in any t-interval in which $I(t) \equiv 1$. We may think of $I(t)$ and $\zeta(t)$ as describing the condition of a *single* particle which can be sometimes a photon ($I = 1$) and sometimes an electron ($I = 2$) and which has an energy $\zeta(t)$. We call $(I(t), \zeta(t))$ the "expectation process," for reasons to be seen below.

If we take $\zeta(0) = 1$ and $I(0) = i$, $i = 1$ or 2, then if $0 < \epsilon < 1$, $(I(t), \zeta(t))$ has for $t > 0$ a probability density $p_{ij}(\epsilon, t)\, d\epsilon$ for the probability that $I(t) = j$ and $\epsilon < \zeta(t) < \epsilon + d\epsilon$. Let $m_{ij}(\epsilon, t)\, d\epsilon$ be the expected number, for the cascade process with $\beta = 0$, of particles of type j at t with energies between ϵ and $\epsilon + d\epsilon$, starting with one particle of type i and energy 1; $i, j = 1$ for photons and 2 for electrons. (These expectation densities are easily seen to exist for $t > 0$, $0 < \epsilon < 1$.)

THEOREM. *We have*[9] $p_{ij}(\epsilon, t) = \epsilon m_{ij}(\epsilon, t)$.

This can be seen by comparing differential equations in t for p_{ij} with those for m_{ij}; the latter can be obtained from equations (2.2) and (2.3) and the corresponding equations for the number of photons. Similarly related Markov processes can, of course, be found for other branching processes.

Since for the expectation process there is, with probability 1, a smallest t where I changes, we have a natural means of using the regeneration-point method to get integral equations for the p_{ij} and hence for the m_{ij}; the equations obtained in this way are different in form from those mentioned at the end of Section 2. Here we merely mention that these integral equations are convenient for proving differentiability properties in ϵ of the m_{ij} or p_{ij}. The correspondence between the two processes might be useful in artificial sampling (Monte Carlo) experiments; using the expectation process to determine p and hence m, one would deal with less complicated histories than if the original process were sampled directly.

4. *Steady States and Limits for the Case $\beta = 0$.*—The process $(I(t), \zeta(t))$ has the stationary "distribution"[10] which assigns the weights $K(a_2 - a_1)$ and $\lambda(a_2 - a_1)$ to the events $\{I(t) = 1, a_1 < -\log \zeta(t) < a_2\}$ and $\{I(t) = 2, a_1 < -\log \zeta(t) < a_2\}$. Hence the original cascade has the stationary densities $K\, d\epsilon/\epsilon^2$ and $\lambda\, d\epsilon/\epsilon^2$ for the expected number of photons or electrons in the energy range ϵ, $\epsilon + d\epsilon$. For particles of a single type, a corresponding result was given by Jánossy.[11]

Next let $z(t)$ be the total energy at t in all electrons, for the cascade process with $\beta = 0$. Since $E[z(t)] = P[I(t) = 2]$, and since $P[I(t) = 2]$ has from the theory of Markov processes the limit $\lambda/(\lambda + K)$ as $t \to \infty$, we have (the symbol E denotes expected value)

$$\lim_{t \to \infty} E[z(t)] = \frac{\lambda}{\lambda + K}, \tag{4.1}$$

a result given by Bhabha and Chakrabarty.[3] We can strengthen equation (4.1) to get convergence in probability also.[12] In fact,

$$\lim_{t \to \infty} E\left[z(t) - \frac{\lambda}{\lambda + K}\right]^2 = 0. \tag{4.2}$$

5. *Cascades with $\beta > 0$.*—Let $\epsilon_0(t)$ be a random function defined as in Section 2; for any $\beta > 0$, define[13]

$$\epsilon_1(t) = \text{Max}\left\{0, \epsilon_0(t)\left[1 - \beta \int_0 \frac{ds}{\epsilon_0(s)}\right]\right\}. \tag{5.1}$$

This formula, which has apparently not been noticed in the literature, gives a representation for the straggling when β is the collision factor. It may be of use in sampling experiments, since it gives a convenient way of constructing sample ϵ_1-functions corresponding to various values of β, when a single sample ϵ_0-function is available.[14]

Now let $N(t)$ be the total number of electrons of all energies at t, electrons whose energies have reached 0 not being counted. It must be recalled that we are using the usual simplifying assumption that the functions q and k of Section 1 do not depend on energy, so that no lower limit is assumed for the energy of an electron, whereas in actuality an electron has a certain minimum (rest) energy. However, even for this simplified model Bhabha and Chakrabarty[3] obtained from the moment equations the result (deducible also from energy considerations) that $E\,[N(t)]$ is finite when $\beta > 0$. We can strengthen this to the result that $E[N^2(t)]$ is also finite when $\beta > 0$. However, a direct analysis of the successive generations of the process shows that if there is one initial electron, then, *with probability* 1 $N(t)$ *reaches* ∞ *in every t-interval.* Although this result must be regarded as a peculiarity of the mathematical model (the so-called "Approximation B"), it suggests that in reality the distribution of the maximum of $N(t)$ over a t-interval may differ markedly from the distribution for fixed t.

[1] H. J. Bhabha and W. Heitler, "Fast Electrons and the Theory of Cosmic Showers," *Proc. Roy. Soc. London, A*, **159**, 432–458, 1937.

[2] J. F. Carlson and J. R. Oppenheimer, "On Multiplicative Showers," *Phys. Rev.*, **51**, 220–231, 1937.

[3] H. J. Bhabha and S. K. Chakrabarty, "The Cascade Theory with Collision Loss," *Proc. Roy. Soc. London, A*, **181**, 267–302, 1943.

[4] "Straggling" is the name applied to the random decrease in energy of an electron.

[5] H. Bethe and W. Heitler, "On the Stopping of Fast Particles and on the Creation of Positive Electrons," *Proc. Roy. Soc. London, A*, **146**, 83–112, 1934.

[6] M. S. Bartlett and D. G. Kendall, "On the Use of the Characteristic Functional in the Analysis of Some Stochastic Processes Occurring in Physics and Biology," *Proc. Cambridge Phil. Soc.*, **47**, 65–76, 1951.

[7] L. Jánossy, "Studies on the Theory of Cascades," *Acta phys. Acad. sci. Hung.*, **2**, 289–333, 1952; this article mentions earlier work of Jánossy.

[8] J. E. Moyal, "Theory of the Ionization Cascade," *Nuclear Physics*, **1**, 180–195, 1956.

[9] Both p and m have versions which are continuous in ϵ; taking these, the equality holds for all, not just almost all, ϵ.

[10] On stationary "distributions" see T. E. Harris, "The Existence of Stationary Measures for Certain Markov Processes," *Proc. Third Berkeley Symposium Math. Statistics and Probability*, **2**, (University of California Press, 1956), 113–124.

[11] L. Jánossy, "On the Absorption of a Nucleon Cascade," *Proc. Roy. Irish Acad., A*, **53**, 181–188, 1950.

[12] The random process $e^{(K+\lambda)t}[z(t) - \lambda/(K + \lambda)]$ is a martingale but possibly not a convergent one; at least its mean square is unbounded at $t \to \infty$. Equation (4.2) can be shown by considering a functional equation for the characteristic function of $z(t)$.

[13] The ϵ_0-process is defined in such a way that with probability 1 the sample functions are positive nonincreasing step functions (although with infinitely many jumps in each t-interval).

[14] For a direct study of the distribution of ϵ_1 see A. Ramakrishnan and P. M. Mathews, "Straggling of the Range of Fast Particles as a Stochastic Process," *Proc. Indian Acad. Sci.*, **41**, 202–209, 1956.

THE IDENTITY OF THE WEAK AND STRONG EXTENSIONS OF A LINEAR ELLIPTIC DIFFERENTIAL OPERATOR

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1. Let Ω be an open subset of the Euclidean space R^n. Let

$$D = \sum_{j_1+\ldots+j_n \leq m} a_{j_1 \ldots j_n}(x) \frac{\partial^{j_1+\ldots+j}}{\partial x_1^{j_1} \ldots \partial x_n^{j_n}}$$

be a linear differential operator in Ω with indefinitely differentiable complex-valued coefficients $a_{j_1 \ldots j_n}(x)$, $x \equiv (x_1, \ldots, x_n) \in \Omega$. D operates on the space of distributions in Ω. Let D_1 denote the restriction of D to the space of C^∞ functions with compact supports. The *weak extension* D_W of D_1 in $L^2(\Omega)$ is defined as follows: the domain M of D_W consists of all those elements f in L^2 for which Df, formed in the sense of distributions, is in L^2, and for such f, $D_W f$ is defined to be Df. The *strong extension* D_S of D_1 in L^2 is the closure in L^2 of the operator D_2, where D_2 is defined as follows: its domain consists of all f in L^2 which are C^∞ and for which Df, formed in the usual sense, belongs to L^2, and for such f, $D_2 f = Df$. It is easily seen that D_W is an extension of D_S. It is not known whether, in general, the weak and strong extensions coincide. In this note we prove their identity for a class of differential operators.[1]

2. Theorem 1. *Let Ω be an arbitrary open subset of R^n, and let D be formally self-adjoint and elliptic.*[2] *The weak and strong extensions of D_1 in $L^2(\Omega)$ coincide.*

Proof: By assumption, D_1 is a symmetric operator in L^2; also, D_W is the adjoint of D_1 in L^2. We have, by a theorem of von Neumann,[3]

$$\text{Domain of } D_W = \text{domain of the closure of } D_1 \oplus E(i) \oplus E(-i), \qquad (1)$$

where $E(i)$ and $E(-i)$ denote the spaces of all square-integrable solutions of the equations $DT = iT$ and $DT = -iT$, respectively. From the results of F. John and L. Schwartz,[2] the elements of $E(i)$ and $E(-i)$ are C^∞ functions. It follows immediately from this and from decomposition (1) that the weak and strong extensions coincide.

3. Theorem 2. *Assume that Ω is bounded. Let D be uniformly elliptic,*[2, 4] *not necessarily formally self-adjoint. Then the weak and strong extensions of D_1 coincide.*

Proof: Since the identity of $(D + \lambda)_S$ and $(D + \lambda)_W$, for λ real, implies that of D_S and D_W, it is sufficient to prove that $(D + \lambda)_S \equiv (D + \lambda)_W$ for some λ. Let $D + \lambda = D'$. The domain M of D'_W is a Hilbert space with the scalar product

$$((f_1, f_2)) = (f_1, f_2)_{L^2} + (D'f_1, D'f_2)_{L^2},$$

and we have to show that $M \cap \mathcal{E}$ is dense in M in this topology, $\mathcal{E}$ denoting the space of C^∞ functions in Ω. Gårding's inequality[2, 4] allows one to define the generalized Friedrich's extension[4-6] of D_1; we denote its domain by N. We consider N as a Hilbert space with the scalar product induced from M. Then, for λ sufficiently large, $D' \mid N : N \to L^2$ is a *topological isomorphism* of N onto L^2.[5, 6] Since C^∞ functions in L^2 are dense in L^2 and since $(D' \mid N)^{-1}$: $L^2 \to N$ maps C^∞ functions into C^∞

functions by the elliptic character of D, it follows therefore that $N \cap \mathcal{E}$ is dense in N. Now if $f \in M$, we can find a unique $f' \in N$ such that $D'f = D'f'$. It follows immediately that

$$M = N \oplus L, \tag{2}$$

where L is the space of all square-integrable solutions of the equation $D'f = 0$. Again, by the elliptic character of D, $L \subset \mathcal{E}$. So, from decomposition (2), we see that $M \cap \mathcal{E}$ is dense in M.

Remark 1: A similar argument shows that if, in Theorem 2, the coefficients are analytic, D_W is the closure of its restriction to analytic functions in the domain of D_W.

Remark 2: Actually, for the proof of Theorem 2, it is sufficient to know that D' maps M *onto* L^2. For, by Banach's open mapping theorem, the map $D'|M : M \to L^2$ will then be an open mapping, and the inverse image of the set $\mathcal{E} \cap L^2$, everywhere dense in L^2, will be everywhere dense in M.

[1] For the case of second-order elliptic operators see M. S. Birman, *Doklady Akad. Nauk S.S.S.R.*, N.S., **92**, 205–208, 1953; and for that of a class of operators with constant coefficients see L. Hörmander, *Acta Math.*, **94**, 241, 1955.

[2] L. Gårding, *Math. Scand.*, **1**, 55–72, 1953.

[3] J. von Neumann, *Math. Ann.*, **102**, 85–86, 1930.

[4] L. Nirenberg, *Communs. Pure and Appl. Math.*, **8**, 648–684, 1955, in particular pp. 657–659.

[5] J. L. Lions, *Acta Math.*, **94**, 26, 1955.

[6] P. D. Lax and A. N. Milgram, "Contributions to the Theory of Partial Differential Equations," *Ann. Math. Studies*, **33**, 167–190, 1954.

ON THE PRINCIPLE OF INVARIANT IMBEDDING AND DIFFUSE REFLECTION FROM CYLINDRICAL REGIONS

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1. *Introduction.*—The importance of various principles of invariance in treating problems of radiative transfer has been discussed by Ambarzumian[1, 2] and Chandrasekhar,[3] who applied these principles to a variety of problems connected with plane-parallel regions. However, the application of these concepts to the corresponding problems for cylindrical and spherical regions is not immediate. Chandrasekhar has observed: "The extension of the approximate methods of solution to other geometries is straightforward; but it is not equally apparent what the general principles are which will play the same unique role as the principle of invariance in plane-parallel atmospheres."[4]

In our first paper[5] we introduced the principle of invariant imbedding as an extension of the ideas of Ambarzumian and Chandrasekhar and utilized this principle to treat the case of inhomogeneous plane-parallel regions. The regions are inhomogeneous in the sense that the phase function, the absorption coefficient and the

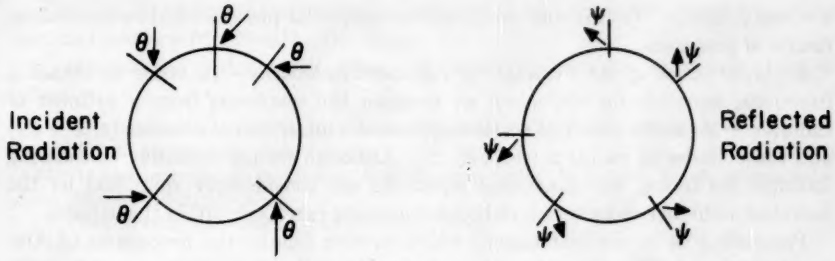

FIG. 1

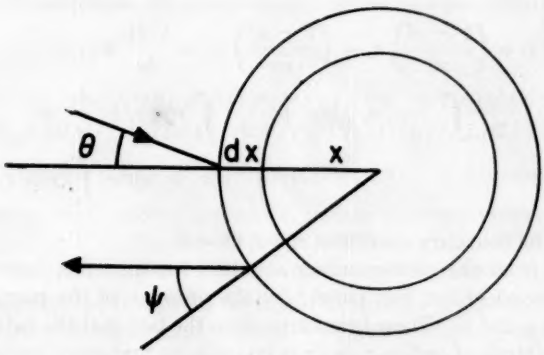

FIG. 2

emission coefficient, to use the terminology of Chandrasekhar, are all allowed to be functions of position. In this paper we propose to show that this new concept enables us to treat cylindrical and, analogously, spherical geometries in a unitary fashion and thus provides an answer to the query of Chandrasekhar. Analytic and computational questions arising from this treatment will be discussed in greater detail in a subsequent publication.

2. *The Physical Model.*—Let us now consider the problem of determining the intensity of diffusely reflected radiation from the surface of a circular cylinder. We assume that the symmetry properties of the medium are such that it suffices to consider interactions in a cross-section perpendicular to the axis, that the absorption and scattering parameters of elemental volumes depend only upon distance from the axis, and, finally, that the scattering is isotropic.

Consider an incoming flux of radiation making a constant angle θ with the radius of the circle, as indicated in Figure 1, and taken to have intensity 1 per unit length normal to the direction of propagation. This is the appropriate definition of flux for circular regions corresponding to the usual one for plane regions. As in the plane case, the ratio of scattering coefficient to absorption coefficient is denoted by $\lambda(x)/(1 - \lambda(x))$, where x is the distance from the center of the circle.

We now consider the intensity of flux of reflected radiation making a fixed angle ψ with the extended radius of a cylinder of radius x, due to an incoming flux at angle θ (Fig. 1). This intensity is considered to be a function $r(v, u, x)$ of $u = \cos\theta$,

$v = \cos\psi$, and x. In this way we imbed the particular process within a continuous family of processes.

3. Application of the Principle of Invariant Imbedding.—In order to obtain a functional equation for $r(v, u, x)$, we consider the scattering from a cylinder of radius $x + dx$ as the result of scattering from the infinitesimal annulus $(x, x + dx)$ and the cylinder of radius x (see Fig. 2). Although we are definitely considering multiple scattering, our functional equations are considerably simplified by the fact that, to higher-order terms, only one scattering can occur within the annulus.

Proceeding as in our first paper,[5] which in turn follows the procedures of Ambarzumian and Chandrasekhar, we obtain the following nonlinear integro-differential equation:[6]

$$r_x + \left(\frac{1}{u} + \frac{1}{v}\right) r + \left(\frac{1 - v^2}{xv}\right) r_v + \left(\frac{1 - u^2}{xu}\right) r_u = \frac{\lambda(x)}{4v} + \frac{\lambda(x)}{2v} \int_0^1 r(w, u, x)dw + \frac{\lambda(x)}{2} \int_0^1 r(v, z, x) \frac{dz}{z} + \lambda(x) \int_0^1 r(w, u, x)dw \int_0^1 r(v, x, z) \frac{dz}{z}, \quad (3.1)$$

together with the boundary condition, $r(v, u, 0) = 0$.

This differs from the corresponding equation for the inhomogeneous parallel-plane case, derived in our first paper,[5] by the presence of the partial derivatives with respect to u and v. These terms arise from the fact that the radiation incident at angle θ on a circle of radius $x + dx$ is incident, to first-order terms, at an angle $x + (\tan\theta)\, dx/x$ on a circle of radius x.

4. Reduction in Dimensionality.—As the equation stands, it is an equation for a function of three variables, $r(v, u, x)$, two spacelike variables, u and v, and one timelike variable, x. Let us now show that this equation may be replaced by two equations for the functions $P(v, x)$ and $Q(u, x)$, defined as

$$P(v, x) = \int_0^1 r(v, z, x) \frac{dz}{z}, \qquad Q(u, x) = \int_0^1 r(w, u, x)\, dw. \quad (4.1)$$

Equation (3.1) has the form

$$L(r) = F(P(v, x), Q(v, x)), \quad (4.2)$$

where L is a linear partial differential operator. Solving for r in terms of F, using characteristic theory or otherwise, we obtain a relation of the form

$$r = \Lambda(F), \quad (4.3)$$

where Λ is a linear operator. Using this relation, we form $P(v, x)$ and $Q(u, x)$ as defined above.

$$P(v, x) = \int_0^1 \Lambda(F) \frac{dz}{z}, \qquad Q(u, x) = \int_0^1 \Lambda(F)\, dw. \quad (4.4)$$

This last set of equations constitutes a system of two nonlinear integral equations for the functions $P(v, x)$ and $Q(u, x)$.

Computational and analytic aspects of these equations will be presented subsequently.

[1] V. A. Ambarzumian, "Diffuse Reflection of Light by a Foggy Medium," *Compt. rend. Acad. Sci. U.R.S.S.*, **38**, 229–232, 1943.

[2] V. A. Ambarzumian, E. R. Mustel', A. B. Severny, and V. V. Sobolev, *Teoreticheskaia Astrofizika* (Moscow, 1952), chap. viii.

[3] S. Chandrasekhar, *Radiative Transfer* (Oxford, 1950).

[4] *Ibid.*, p. 364.

[5] R. Bellman and R. Kalaba, "On the Principle of Invariant Imbedding and Propagation through Inhomogeneous Media," these PROCEEDINGS, **42**, 629–632, 1956.

[6] To simplify the analysis, we are considering only the case of spherinal scattering here.

ON THE PRINCIPLE OF INVARIANT IMBEDDING AND ONE-DIMENSIONAL NEUTRON MULTIPLICATION

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1. *Introduction.*—In this paper we wish to introduce a new method of treating problems involving neutron multiplication by fission, with special regard to questions of critical mass and distribution of neutrons. Our results derive from applications of the principle of invariant imbedding, which we have introduced and discussed in two previous papers,[1, 2] the first of which contains a brief discussion of the close interrelation between the method we employ here and the point-of-regeneration technique employed in Bellman and Harris.[3]

In order to present the essentials of the technique, as free as possible of analytic difficulties, we shall consider first only one-dimensional flux of neutrons. This case is of interest in itself, from both the physical and the mathematical points of view.

We shall consider first a one-velocity case, deriving partial differential equations for the generating functions of the probability distribution of "reflected" and "transmitted" neutrons (those terms will be defined precisely below) and ordinary differential equations for the moments. Although the moment equations can be used to determine critical length, an inspection of the first and second moments shows that these yield only a very imperfect picture of the actual distribution of neutrons. Classical treatment is based on expected values.

We then turn to a two-velocity case, to illustrate the applicability of our methods to the more realistic situations where fission and fission products are energy-dependent, as well as situations where several types of particles are involved.

It will be clear from our discussion and from previous papers how our procedure can be modified to take into account absorption of neutrons and inhomogeneity of the medium.

In subsequent papers we shall first consider the dependence on time of the dis-

tribution of neutrons and the case of continuous dependence on energy for the one-dimensional case and then turn to a discussion of the two-dimensional plane slab, the cylindrical case, and the spherical case.

2. *Description of the Process.*—Consider a finite one-dimensional "rod," the interval $[x, 0]$, constituted of a material with the property that a neutron traversing an infinitesimal interval, $[y + dy, y]$, has, to first-order terms, the probability $a\,dy$ of splitting into two neutrons, of the same type as the original, one going to the left and one to the right.

Assuming that a neutron, the "trigger" neutron, enters from the left, as diagramed in Figure 1, we wish to determine the number of neutrons emerging from the left ("reflected" neutrons) and the number of neutrons emerging from the right ("transmitted" neutrons).

FIG. 1

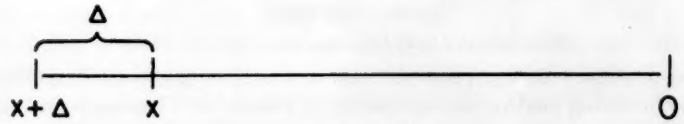

FIG. 2

3. *Mathematical Formulation.*—Let us define the two sequences of probabilities $\{p_n(x)\}, \{q_n(x)\}$, as follows:

$$\begin{aligned} p_n(x) &= \text{the probability that } n \text{ particles will be reflected from an interval of length } x, \text{ over all time, as a result of one neutron entering at } x \text{ at time 0.} \\ q_n(x) &= \text{the probability that } n \text{ particles will be transmitted through an interval of length } x, \text{ over all time, as a result of one neutron entering at } x \text{ at time 0.} \end{aligned} \tag{3.1}$$

The essence of the technique of invariant imbedding is to regard the process described above as a member of a family of processes of the same type. The mathematical statement of this invariance will yield a set of equations governing the process. This is accomplished readily in this case by regarding x as a parameter capable of assuming all positive values.

4. *Basic Functional Equations.*—Let us begin by deriving relations connecting members of the sequences $\{p_n(x + \Delta)\}$ and $\{p_n(x)\}$ for small Δ. Referring to Figure 2, we see that, to first-order terms,

$$p_0(x + \Delta) = (1 - a\Delta)p_0(x). \tag{4.1}$$

To obtain the equations for $p_n(x + \Delta)$, we consider reflection of n neutrons from $[x + \Delta, 0]$ as composed of the following occurrences:

a) Transmission of the neutron through $[x + \Delta, x]$, reflection of n neutrons from $[x, 0]$, transmission of n neutrons through $[x, x + \Delta]$.
b) Transmission of the neutron through $[x + \Delta, x]$, reflection of k neutrons from $[x, 0]$, $k = 1, 2, \ldots, n$, fission of one of these k neutrons in $[x, x + \Delta]$, reflection of $(n - k)$ additional neutrons from $[x, 0]$. (4.2)
c) Fission of the neutron in $[x + \Delta, x]$, reflection of $(n - 1)$ neutrons from $[x, 0]$.

This verbal statement is equivalent to the mathematical relation

$$p_n(x + \Delta) = (1 - a\Delta)\,[p_n(x)\,(1 - a\Delta)^n + a\Delta \sum_{k=1}^{n} k p_k(x) p_{n-k}(x)] + a\Delta p_{n-1}(x), \tag{4.3}$$

to first-order terms in Δ.[4]

Letting $\Delta \to 0$, the limiting equations are

$$p_0'(x) = -a p_0(x),$$
$$p_n'(x) = -(n + 1)a p_n(x) + a p_{n-1}(x) + a \sum_{k=1}^{n} k p_k(x) p_{n-k}(x), \qquad n \geq 1. \tag{4.4}$$

Similarly, we obtain the recurrence relations

$$q_n'(x) = -anq_n(x) + a \sum_{k=1}^{n} k q_k(x) p_{n-k}(x), \qquad n = 1, 2, \ldots, \tag{4.5}$$

with $q_0(x) = 0$.

5. *Partial Differential Equation for Generating Functions.*—Let us define

$$u(x, r) = \sum_{n=0}^{\infty} p_n(x) r^n, \qquad v(x, r) = \sum_{n=1}^{\infty} q_n(x) r^n. \tag{5.1}$$

The recurrence relations of equations (4.4) and (4.5) yield the equations

$$\begin{aligned} u_x &= au(r - 1) + aru_r(u - 1), \\ v_x &= ar(u - 1)v_r. \end{aligned} \tag{5.2}$$

These equations can be explicitly resolved using the method of characteristics. The details are not trivial, however. We shall discuss these matters in detail subsequently.

6. *Critical Length and Moment Equation.*—The concept of "critical length" can be introduced as the smallest value of x for which the expected number of reflected and transmitted neutrons is infinite.

Setting

$$U(x) = u_r\big|_{r=1}, \qquad V(x) = v_r\big|_{r=1}, \tag{6.1}$$

we obtain from equations (5.2), via differentiation and setting $r = 1$, the equations

$$U'(x) = a(1 + U^2), \qquad V'(x) = aUV, \tag{6.2}$$

with $U(0) = 0$, $V(0) = 1$. From these equations we obtain $U(x) = \tan ax$, $V(x) = \sec ax$, showing that the critical length is $\pi/2a$.

In a similar fashion, we find the differential equation for the second moment,

$$\frac{dU_2}{dx} = 2aU + 2aU^2 + 3aUU_2, \qquad U_2(0) = 0, \tag{6.3}$$

which shows that $U_2 = 0(1/(\cos ax)^3)$ as $x \to \pi/2a$. It follows that the expected number of neutrons is an unreliable measure of the nature of the distribution for x near $\pi/2a$.

7. *Two-Velocity Case.*—Following the usual approximation to the physical situation where a neutron possesses a direction and an energy varying continuously over certain limits, let us assume that there are just two types of neutrons, "fast" and "slow," and that in the fission process either can give rise to the other.

To simplify the equations, let us assume that fast neutrons have a probability $a_F dy$ of splitting in $\{y + dy, y\}$ and that, when they do split, there is a probability $1/2$ of a fast neutron produced going to the right and a slow neutron produced going to the left, and probability $1/2$ of a slow neutron going to the right and a fast neutron to the left. Let the same situation prevail with slow neutrons with a_F replaced by a_S.

Let

$$p_{mn}^{(F)}(x) = \text{the probability that } m \text{ fast neutrons and } n \text{ slow neutrons will be reflected from an interval of length } x, \text{ overall time, as a result of one fast neutron entering at } x \text{ at time 0, with } p_{mn}^{(S)}(x) \text{ defined similarly for a slow trigger neutron.} \tag{7.1}$$

Then, as above, we have

$$\begin{aligned} p_{mn}^{(F)}(x + \Delta) = {} & (1 - a\Delta)[p_{mn}^{(F)}(x)\,(1 - a\Delta)^{m+n} + \Sigma p_{k,l}^{(F)}(x)\,[1/2 p_{m-k,\,n-l}^{(F)}(x) \\ & + k a_F 1/2 p_{m-k,\,n-1}(x)]] \\ & + a\Delta[1/2[p_{m-1,\,n}^{(F)}(x) + p_{m,\,n-1}^{(S)}(x)]], \end{aligned} \tag{7.2}$$

with a similar equation for $p_{mn}^{(S)}(x, + \Delta)$.

Passing to the limit, we obtain recurrence relations which permit us to obtain partial differential equations for the generating functions, $u^{(F)}(x, r, t) = \Sigma p_{mn}^{(F)}(x) r^m t^n$ and $u^{(S)}(x, r, t) = \Sigma p_{mn}^{(S)}(x) r^m t^n$. From these we obtain ordinary differential equations for the moments and thus can determine critical length. At present we have not been able to solve these equations explicitly, though numerical solution would be straightforward.

[1] R. Bellman and R. Kalaba, "On the Principle of Invariant Imbedding and Propagation through Inhomogeneous Media," these PROCEEDINGS, **42**, 629–632, 1956.

[2] R. Bellman and R. Kalaba, "On the Principle of Invariant Imbedding and Diffuse Reflection from Cylindrical Regions" (Paper P-976 [Rand Corporation, 1956]).

[3] R. Bellman and T. E. Harris, "On Age-dependent Binary Branching Processes," *Ann. Math.* **55**, 280–295, 1952. For a thorough discussion of branching processes see T. E. Harris, "Some Mathematical Models for Branching Processes," *Proc. Second Berkeley Symposium Math. Statistics and Probability* (Berkeley: University of California Press, 1951), pp. 305–328.

[4] This is equivalent to the fact that in an *infinitesimal* interval it is sufficient to consider only first-order processes.

ON THE EXPANSION OF FUNCTIONS WHICH SATISFY FOUR BOUNDARY CONDITIONS

By S. Chandrasekhar and W. H. Reid

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Communicated April 27, 1957

1. *Introduction.*—In the solution of many problems in applied mathematics it is often convenient to have expansions for functions which vanish, together with their derivatives, at the end points. In some recent work on hydrodynamic and hydromagnetic stability we have found certain types of expansions for such functions which have proved to be very useful and which appear to be novel in this connection. In this paper two types of such expansions will be considered, and the principles underlying them will be described.

2. *Fourier-Type Expansions.*—Consider the characteristic-value problem defined by the equation

$$\frac{d^4y}{dx^4} = \lambda^4 y \tag{1}$$

and the boundary conditions

$$y = 0 \quad \text{and} \quad \frac{dy}{dx} = 0 \quad \text{at} \quad x = \pm\frac{1}{2}. \tag{2}$$

Let λ_m and λ_n denote two distinct characteristic values; then, by using the boundary conditions (2), it can readily be shown that

$$\lambda_m{}^4 \int_{-1/2}^{+1/2} y_m y_n \, dx = \int_{-1/2}^{+1/2} \frac{d^2y_m}{dx^2}\frac{d^2y_n}{dx^2} dx. \tag{3}$$

By subtracting from this equation the corresponding equation with m and n interchanged, we obtain

$$(\lambda_m{}^4 - \lambda_n{}^4) \int_{-1/2}^{+1/2} y_m y_n \, dx = 0; \tag{4}$$

and hence

$$\int_{-1/2}^{+1/2} y_m y_n \, dx = 0 \quad \text{if} \quad m \neq n. \tag{5}$$

The solutions y_n form, therefore, a complete orthogonal set of functions. We shall now obtain the explicit form of these functions.

The solutions of equation (1) fall into two noncombining groups: the even and the odd solutions, with cosine- and sine-like behavior, respectively. Considering first the even solutions of equation (1), we obtain the eigenfunctions

$$C_n(x) = \frac{\cosh \lambda_n x}{\cosh (\lambda_n/2)} - \frac{\cos \lambda_n x}{\cos (\lambda_n/2)}, \tag{6}$$

where the eigenvalues λ_n are the positive roots of the equation

$$\tanh (\lambda/2) + \tan (\lambda/2) = 0; \tag{7}$$

the corresponding odd solutions of equation (1) lead to the eigenfunctions

$$S_n(x) = \frac{\sinh \mu_n x}{\sinh (\mu_n/2)} - \frac{\sin \mu_n x}{\sin (\mu_n/2)}, \tag{8}$$

where the eigenvalues μ_n are the positive roots of the equation

$$\tanh (\mu/2) - \tan (\mu/2) = 0. \tag{9}$$

We shall presently see that, when chosen in this manner, the functions C_n and S_n are normalized to unity. The first few roots of the characteristic equations (7) and (9) have been computed by Rayleigh[1] and are given in Table 1 for convenience. For large values of n, $\lambda_n \to (2n - 1/2)\pi$ and $\mu_n \to (2n + 1/2)\pi$.

TABLE 1

n	λ_n	μ_n
1	4.7300408	7.8532046
2	10.9956078	14.1371655
3	17.2787596	

The functions $C_n(x)$ and $S_n(x)$ defined by equations (6) and (8) have the properties

$$\int_{-1/2}^{+1/2} C_m(x)\, C_n(x)\, dx = \int_{-1/2}^{+1/2} S_m(x)\, S_n(x)\, dx = \delta_{mn} \tag{10}$$

and

$$\int_{-1/2}^{+1/2} C_m(x)\, S_n(x)\, dx = 0. \tag{11}$$

They form, therefore, a complete set of orthonormal functions in the interval $(-1/2, +1/2)$. Thus, if $F(x)$ satisfies the boundary conditions (2), then it can be expanded in the form

$$F(x) = \sum_{n=1}^{\infty} [A_n C_n(x) + B_n S_n(x)], \tag{12}$$

where

$$A_n = \int_{-1/2}^{+1/2} F(x)\, C_n(x)\, dx \qquad \text{and} \qquad B_n = \int_{-1/2}^{+1/2} F(x)\, S_n(x)\, dx.$$

If $F(x)$ is an even function, then $B_n \equiv 0$, corresponding to the familiar Fourier cosine expansion; conversely, if $F(x)$ is odd, then $A_n \equiv 0$, corresponding to the Fourier sine expansion.

3. *An Application to Hydrodynamic Stability.*—To illustrate the use of these functions in problems of hydrodynamic stability, consider the classical Bénard problem of thermal instability in a layer of fluid heated from below, for which the disturbance equation is

$$(D^2 - a^2)^3 v = -a^2 R v, \tag{13}$$

where $D = d/dx$ and R is the Rayleigh number. For two rigid bounding surfaces the boundary conditions which v must satisfy are

$$v = (D^2 - a^2)v = D(D^2 - a^2)v = 0 \qquad \text{at } x = \pm\frac{1}{2}. \tag{14}$$

Now Chandrasekhar[2] has shown that two variational principles exist for this problem, one of which can be described briefly as follows.

By letting

$$(D^2 - a^2)v = G, \tag{15}$$

we can rewrite equation (13) in the form

$$(D^2 - a^2)^2 G = -a^2 R v, \tag{16}$$

while the boundary conditions (14) require that

$$v = G = DG = 0 \qquad \text{at } x = \pm\frac{1}{2}. \tag{17}$$

The characteristic value, R, can then be expressed as the ratio of two positive definite integrals in the form

$$R = \frac{\int_{-1/2}^{+1/2} [(D^2 - a^2)G]^2 \, dx}{a^2 \int_{-1/2}^{+1/2} [(Dv)^2 + a^2 v^2] \, dx}. \tag{18}$$

Thus, if one assumes a form for G which satisfies the boundary conditions $G = DG = 0$ at $x = \pm 1/2$ and *solves* equation (15) subject to the boundary conditions $v = 0$ at $x = \pm 1/2$, then all the boundary conditions of the problem are satisfied and the minimum value of R (with respect to a) can then be obtained from equation (18).

Now it is known that instability first arises in this problem with respect to a symmetric disturbance. Accordingly, to test the method, we assume the very simple trial function $G = C_1(x)$, with no variational parameters, for which the solution of equation (15) is

$$v = -\frac{2\lambda_1^2}{\lambda_1^4 - a^4} \frac{\cosh ax}{\cosh (a/2)} + \frac{1}{\lambda_1^2 - a^2} \frac{\cosh \lambda_1 x}{\cosh (\lambda_1/2)} + \frac{1}{\lambda_1^2 + a^2} \frac{\cos \lambda_1 x}{\cos (\lambda_1/2)}. \tag{19}$$

Equation (18) then leads to the result

$$R = I_1/a^2 I_2, \tag{20}$$

where

$$I_1 = \lambda_1^4 + 2\lambda_1^2 a^2 [\tanh^2 (\lambda_1/2) - (2/\lambda_1) \tanh (\lambda_1/2)] + a^4 \tag{20'}$$

and

$$I_2 = \frac{8\lambda_1^4}{(\lambda_1^4 - a^4)^2} [\lambda_1 \tanh (\lambda_1/2) - a \tanh (a/2)] + \frac{\lambda_1^2}{\lambda_1^4 - a^4} [\tanh^2 (\lambda_1/2) - (2/\lambda_1) \tanh (\lambda_1/2)] - \frac{a^2}{\lambda_1^4 - a^4}, \tag{20''}$$

which gives $R = 1{,}719.1$ for $a = 3.10$. The other variational method,[2] which requires the solution of a fourth-order equation, gives $R = 1{,}715.1$ for $a = 3.12$. Compared with the exact value, $R = 1{,}707.8$ for $a = 3.12$, both methods give very good results. The particular advantage of the present method lies in its greater simplicity with little sacrifice in accuracy.

Expansion in terms of the functions C_n and S_n can also be used for the solution of the problem of the stability of viscous flow between rotating cylinders (cf. Chandrasekhar[3]). Thus, instead of transforming equation (10) (in the paper just referred to) to the form (17) and expanding W in a sine series, we can seek a solution

of equation (10) directly by expanding $(D^2 - a^2)v$ in C_n and S_n, solving for v, and then proceeding to the solution of the characteristic-value problem; for many problems, a direct expansion in terms of C_n and S_n is preferable to one which requires a solution of a higher-order equation.

4. *Fourier-Bessel-Type Expansions.*—For problems involving circular boundaries one must consider the solutions of the equation

$$\left(\frac{d^2}{dr^2} + \frac{1}{r}\frac{d}{dr} - \frac{\nu^2}{r^2}\right)^2 y = \alpha^4 y \tag{21}$$

which satisfy the boundary conditions

$$y = 0 \quad \text{and} \quad \frac{dy}{dr} = 0 \quad \text{at } r = 1 \text{ and } \eta\ (<1). \tag{22}$$

To show that these solutions form an orthogonal set, we proceed as follows. Letting

$$\mathfrak{D} = \frac{d^2}{dr^2} + \frac{1}{r}\frac{d}{dr} - \frac{\nu^2}{r^2}, \tag{23}$$

we can show that

$$\int_\eta^1 y\, \mathfrak{D}f\, r\, dr = \int_\eta^1 f\, \mathfrak{D}y\, r\, dr \tag{24}$$

for an arbitrary f, if y and dy/dr vanish at the end points. Thus, from equation (21), we have

$$\alpha_m{}^4 \int_\eta^1 y_m y_n\, r\, dr = \int_\eta^1 y_n\, \mathfrak{D}^2 y_m\, r\, dr = \int_\eta^1 \mathfrak{D}y_n\, \mathfrak{D}y_m\, r\, dr. \tag{25}$$

By subtracting from this equation the corresponding equation with m and n interchanged, we obtain

$$(\alpha_m{}^4 - \alpha_n{}^4) \int_\eta^1 y_m y_n\, r\, dr = 0; \tag{26}$$

and hence

$$\int_\eta^1 y_m y_n\, r\, dr = 0 \qquad \text{if } m \neq n. \tag{27}$$

The solutions y_n form, therefore, a complete orthogonal set. We shall proceed to obtain the associated characteristic equation and the explicit form of the eigenfunctions.

The general solution of equation (21) is

$$y = AJ_\nu(\alpha r) + BY_\nu(\alpha r) + CI_\nu(\alpha r) + DK_\nu(\alpha r), \tag{28}$$

where A, B, C, and D are arbitrary constants and the definitions of the Bessel functions are those of Watson.[4] In writing the solution in the foregoing form, we have assumed that ν is an integer; if ν should not be an integer, we need only write $J_{-\nu}$ in place of Y_ν to obtain the corresponding solution.

For purposes of applying the boundary conditions, it is convenient to note that

$$\frac{1}{\alpha r^\nu}\frac{d}{dr}(r^\nu y) = AJ_{\nu-1}(\alpha r) + BY_{\nu-1}(\alpha r) + CI_{\nu-1}(\alpha r) - DK_{\nu-1}(\alpha r). \tag{29}$$

The boundary conditions (18) then yield the equations

$$\begin{aligned} AJ_\nu(\alpha) + BY_\nu(\alpha) + CI_\nu(\alpha) + DK_\nu(\alpha) &= 0, \\ AJ_\nu(\alpha\eta) + BY_\nu(\alpha\eta) + CI_\nu(\alpha\eta) + DK_\nu(\alpha\eta) &= 0, \\ AJ_{\nu-1}(\alpha) + BY_{\nu-1}(\alpha) + CI_{\nu-1}(\alpha) - DK_{\nu-1}(\alpha) &= 0, \\ AJ_{\nu-1}(\alpha\eta) + BY_{\nu-1}(\alpha\eta) + CI_{\nu-1}(\alpha\eta) - DK_{\nu-1}(\alpha\eta) &= 0, \end{aligned} \tag{30}$$

so that the characteristic values α_n are the roots of the equation

$$\begin{vmatrix} J_\nu(\alpha) & Y_\nu(\alpha) & I_\nu(\alpha) & K_\nu(\alpha) \\ J_\nu(\alpha\eta) & Y_\nu(\alpha\eta) & I_\nu(\alpha\eta) & K_\nu(\alpha\eta) \\ J_{\nu-1}(\alpha) & Y_{\nu-1}(\alpha) & I_{\nu-1}(\alpha) & -K_{\nu-1}(\alpha) \\ J_{\nu-1}(\alpha\eta) & Y_{\nu-1}(\alpha\eta) & I_{\nu-1}(\alpha\eta) & -K_{\nu-1}(\alpha\eta) \end{vmatrix} = 0. \tag{31}$$

The corresponding eigenfunctions then become

$$y_n = J_\nu(\alpha_n r) + B_n Y_\nu(\alpha_n r) + C_n I_\nu(\alpha_n r) + D_n K_\nu(\alpha_n r), \tag{32}$$

where we have taken $A = 1$ for convenience.

The first few roots of equation (31) for $\nu = 1$ are given in Table 2 for three values of η, together with the corresponding values of B_n, C_n, and D_n, and of the normalizing integral, N_n, the explicit form of which we shall now derive. For this latter purpose, consider the quantities

$$u(r) = AJ_\nu(\alpha r) + BY_\nu(\alpha r) \tag{33}$$

and

$$v(r) = CI_\nu(\alpha r) + DK_\nu(\alpha r), \tag{34}$$

which satisfy the equations

$$\frac{1}{r}\frac{d}{dr}\left(r\frac{du}{dr}\right) + \left(\alpha^2 - \frac{\nu^2}{r^2}\right)u = 0 \tag{35}$$

and

$$\frac{1}{r}\frac{d}{dr}\left(r\frac{dv}{dr}\right) - \left(\alpha^2 + \frac{\nu^2}{r^2}\right)v = 0. \tag{36}$$

TABLE 2

THE ROOTS OF EQUATION (31) FOR $\nu = 1$ AND RELATED QUANTITIES

	$\eta = 1/4$	$\eta = 1/2$	$\eta = 3/4$
α_1	$+6.39034$	$+9.49896$	$+1.89344 \times 10^{1}$
N_1	$+1.34093$	$+1.42107$	$+4.5610 \times 10^{-1}$
B_1	-5.87014	-9.07642	-1.02780×10^{1}
C_1	-1.49706×10^{-2}	$+1.00635 \times 10^{-3}$	-8.91136×10^{-8}
D_1	-1.07799×10^{1}	$+4.39637 \times 10^{2}$	-6.65593×10^{6}
α_2	$+1.05517 \times 10^{1}$	$+1.57397 \times 10^{1}$	
N_2	$+2.68344 \times 10^{-2}$	$+3.0630$	
B_2	-4.28052×10^{-1}	-1.73711×10^{1}	
C_2	$+4.16730 \times 10^{-5}$	$+3.67889 \times 10^{-6}$	
D_2	-5.98695	-1.95527×10^{4}	
α_3	$+1.47290 \times 10^{1}$	$+2.20178 \times 10^{1}$	
N_3	$+2.45037 \times 10^{-2}$	$+3.65915$	
B_3	$+7.12511 \times 10^{-1}$	-2.24667×10^{1}	
C_3	-7.14451×10^{-7}	$+8.86448 \times 10^{-9}$	
D_3	-1.99016×10^{1}	$+5.91172 \times 10^{5}$	

Consider now the integral

$$\int_\eta^1 r(u+v)^2\,dr = \int_\eta^1 r(u^2+v^2)\,dr + 2\int_\eta^1 ruv\,dr. \tag{37}$$

Multiplying equation (35) by rv and equation (36) by ru and subtracting the results, we obtain

$$2\alpha^2 \int_\eta^1 ruv\, dr = \int_\eta^1 \left[u \frac{d}{dr}\left(r \frac{dv}{dr} \right) - v \frac{d}{dr}\left(r \frac{du}{dr} \right) \right] dr = \left[r \left(u \frac{dv}{dr} - v \frac{du}{dr} \right) \right]_\eta^1. \tag{38}$$

Since $u(r) + v(r)$ and $u'(r) + v'(r)$ vanish at the end points, the right-hand side of this equation vanishes also; equation (37) therefore reduces to

$$\int_\eta^1 r(u + v)^2\, dr = \int_\eta^1 r(u^2 + v^2)\, dr. \tag{39}$$

To evaluate the integrals on the right-hand side of this equation, consider first the differential equation satisfied by v. If this equation is multiplied by $2rv'(r)$, then one obtains a result that can be written in the form

$$\frac{d}{dr}\left(r \frac{dv}{dr} \right)^2 = \alpha^2 r^2 \frac{d}{dr} v^2 + \nu^2 \frac{d}{dr} v^2; \tag{40}$$

and when this result is integrated between η and 1, we have

$$2\alpha^2 \int_\eta^1 rv^2\, dr = \left[-r^2 \left(\frac{dv}{dr} \right)^2 + (\alpha^2 r^2 + \nu^2) v^2 \right]_\eta^1. \tag{41}$$

Similarly,

$$2\alpha^2 \int_\eta^1 ru^2\, dr = \left[r^2 \left(\frac{du}{dr} \right)^2 + (\alpha^2 r^2 - \nu^2) u^2 \right]_\eta^1; \tag{42}$$

hence

$$2\alpha^2 \int_\eta^1 r(u^2 + v^2)\, dr = \left[r^2 \left\{ \left(\frac{du}{dr} \right)^2 - \left(\frac{dv}{dr} \right)^2 \right\} + \alpha^2 r^2 (u^2 + v^2) + \nu^2 (u^2 - v^2) \right]_\eta^1. \tag{43}$$

Since $u = -v$ and $u' = -v'$ at the end points, this reduces to the very simple result

$$\int_\eta^1 r(u^2 + v^2)\, dr = [r^2 u^2]_\eta^1 = [r^2 v^2]_\eta^1 = u^2(1) - \eta^2 u^2(\eta). \tag{44}$$

Thus the normalizing integral for y_n can be written in the form

$$\int_\eta^1 r y_n^2\, dr = N_n, \tag{45}$$

where

$$N_n = [J_\nu(\alpha_n) + B_n Y_\nu(\alpha_n)]^2 - \eta^2 [J_\nu(\alpha_n \eta) + B_n Y_\nu(\alpha_n \eta)]^2, \tag{46}$$

the numerical values of which are given in Table 2 for $\nu = 1$.

A function $F(r)$ which satisfies the boundary conditions (22) can therefore be expanded in the form

$$F(r) = \sum_{n=1}^{\infty} A_n y_n(r), \qquad \text{where} \qquad A_n = \frac{1}{N_n} \int_\eta^1 F(r)\, y_n(r)\, r\, dr, \tag{47}$$

and $y_n(r)$ are the eigenfunctions defined by equation (32) in close analogy with the usual Fourier-Bessel expansions.

5. *Concluding Remarks.*—As we have indicated in the Introduction, expansions of the type considered here should prove useful in the solution of many hydrodynamic and hydromagnetic problems. In particular, the classical problems of the stability of viscous flow between rotating cylinders[5] and in a curved channel,[6] which have never been solved when the spacing between the cylinders is comparable with their radii, can be treated by this method.

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*ELECTROMAGNETIC DETERMINATION OF REGIONAL BLOOD FLOW IN UNANESTHETIZED ANIMALS**

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The interest in a method for continuous registration of blood flow in essentially normal conscious animals goes beyond the objective of studying flow as a circulatory parameter. By far the greatest utility of such a method would be in permitting the study of the activity of a great variety of organs by one single method. The rate of blood supply to an organ is an important parameter, revealing its rate of activity, which determines the required rate of supply of oxygen, hormones, and nutrient materials as well as the rate of removal of products of metabolism. The blood flow through an organ, when correlated with its state of activity, could be used to follow the variations in the activity of the organ. Thus it may become possible,

in studies aiming at initial detection of the effectiveness of various stimulating and inhibiting factors affecting various organs, to replace discontinuous and often cumbersome chemical tests of secretory or excretory functions of these organs, used as an index of their activity, by a continuous record of blood flow. The applicability of such studies to the solution of pharmacological and physiological problems as well as to investigations in experimental pathology is obvious.

An ideal method for the determination of regional blood flow in normal animals should have the following characteristics: it should measure flow through intact blood vessels; the blood vessel should be left in its normal place and not be exposed; the measuring device and procedure should be such that drugs (such as heparin or anesthetics) would not be needed, the animal being as nearly as possible in its normal state; the inserted instruments should be suitable for chronic implantation; the relation between blood flow and instrument reading should be linear and independent of variations in phyiscal factors like temperature, viscosity, velocity profile (laminar or turbulent flow), etc.; the response of the instrument to velocity changes should be instantaneous; and frequent calibrations of the instrument should not be necessary. It is believed that the method to be described below is the nearest approach so far to these desired ideal conditions.

1. *Principle and Development of the Method.*—The principle of the method is based on the phenomenon of electromagnetic induction discovered by Faraday. Faraday demonstrated the generation of electromotive forces (emf's) in closed electric circuits traversed by a varying magnetic flux, as well as in solid conductors moving at right angles to a magnetic field (Faraday's unipolar inductor). Faraday could easily have demonstrated, by means available in his laboratory, the induction of an emf in a liquid traversing a magnetic field, by allowing mercury to flow through a pipe disposed at right angles to the magnetic field and by picking up the voltage induced in the moving liquid by means of two electrodes disposed at the ends of the pipe diameter perpendicular to the magnetic lines of force (Fig. 1*A*). Strangely enough, Faraday did not perform such an experiment but, instead, spent a considerable amount of time conducting experiments at the Waterloo Bridge in London, trying to demonstrate the induction of an electromotive force in the River Thames as its water moves across the magnetic field of the earth. Owing to difficulties caused by polarization of the electrodes, Faraday finally gave up these experiments. This negative result is mentioned by him in a Bakerian lecture.[1]

The qualitative idea of the possibility of inducing an emf in a moving fluid, which Faraday evidently conceived, is, owing to complications introduced by the velocity distribution in the flowing fluid, still considerably removed from the realization of the possibility of quantitative measurement of flow by electromagnetic induction. The importance of this factor was realized by Williams,[2] who, in fact, used electromagnetic induction for an indirect determination of the velocity distribution in pipes, using a special nonpolarizable system (copper electrodes in a copper sulfate solution). Although Williams' results imply the possibility of development of a flowmeter based on electromagnetic induction, there is no such suggestion made in his paper. That paper was not followed by further publications on this subject.

Unaware of the work of Williams and Faraday, Fabre proposed in a letter to the French Academy of Sciences[3] a qualitative biological use ("un hémodromograph sans palette") for electromagnetic induction. His note suggested the possibility of

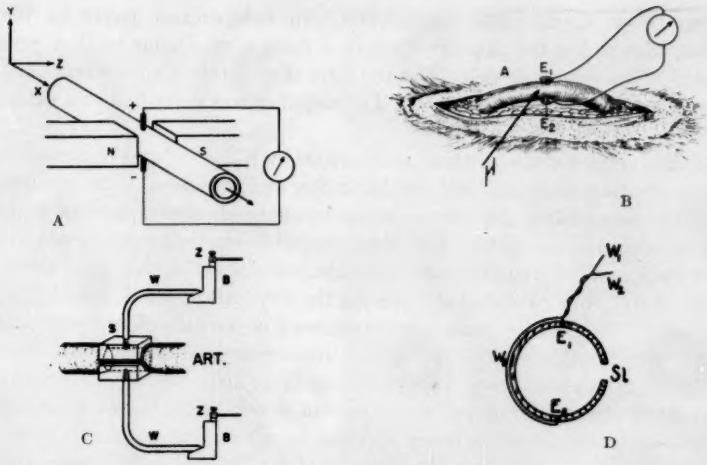

FIG. 1*A*.—Scheme of electromagnetic flowmeter. *N* and *S*, pole pieces of magnet; + and −, electrodes which pass through the pipe wall to make contact with the fluid.

FIG. 1*B*.—Scheme for determination of blood flow in intact artery. *H*, magnetic field vector; *A*, artery; E_1 and E_2, nonpolarizable electrodes touching the artery wall.

FIG. 1*C*.—Pickup sleeve *S* with artery and nonpolarizable electrodes. *W*, wicks soaked in agar-saline; *B*, porous clay boots filled with zinc sulfate; *Z*, amalgamated Zn electrodes.

FIG. 1*D*.—Pickup sleeve with metal electrodes. *Sl*, slit through which artery is slipped into the sleeve channel; E_1 and E_2, pickup electrodes contacting the artery; W_1 and W_2, electrode lead wires.

detecting variations in flow of blood through a pipe equipped with electrodes and tied between the two parts of a cut blood vessel. His discussion was qualitative, and no experimental evidence was presented. The instrumentation described by him could not have permitted measurement of the rate of flow, which evidently was not attempted. This note was not followed by further publications on this subject.

The above publications have had no effect on the development of the electromagnetic flowmeter. The method was developed independently by investigators who have been unaware of the close approaches of the preceding authors. In 1936 it was demonstrated[4] that electromagnetic induction could be used for quantitative determination of fluid flow in pipes, and the anticipated linear relation between the volumetric rate of flow and induced voltage was demonstrated. Furthermore, it was shown that flow of blood through arteries could be recorded without injuring the artery wall, merely by applying nonpolarizable electrodes to the outside of the walls of the artery at the ends of its diameter perpendicular to the magnetic field (*H*) (Fig. 1*B*). At the same time, the instantaneous response of the apparatus to rapidly pulsating flow was demonstrated, and the average rate of flow of blood through a dog's carotid artery was determined. The instrumentation used in this communication was very simple, consisting of a d'Arsonval galvanometer for average-flow measurement and a string galvanometer for recording of instantaneous flow. In the subsequent communications[5–7] the use of a chopper amplifier was described, and a sleeve was introduced for convenient application of nonpolarizable electrodes (Fig. 1*C*).

Shortly after Kolin's 1936 publication,[4] an independent paper by Wetterer appeared,[8] describing the same method in a form very similar to that presented by Kolin. Experimental evidence in the form of calibration curves and blood-flow records was presented. The use of a d.c. amplifier was described in a subsequent paper.[9]

The initial version of the method, as described by Kolin[4, 5, 7] and Katz and Kolin,[6] utilizes a constant magnetic field, so that a d.c. emf is induced with unidirectional flow. This necessitates the use of nonpolarizable electrodes and of a suitable system of d.c. amplification. The nonpolarizable electrodes are quite cumbersome in use,[10] and d.c. amplifiers are, as a rule, less stable than a.c. amplifiers. The next logical step thus consisted of replacing the constant magnetic field by an alternating one.[11] In this case, an a.c. emf is induced in the case of unidirectional flow, so that nonpolarizable electrodes become unnecessary, ordinary metal electrodes touching the artery wall being quite adequate (Fig. 1*D*). An a.c. amplifier can be used to detect the signal induced due to flow of blood. It is, however, necessary to use a special compensator,[12] in order to adjust for a zero reading at zero flow. Such zero adjustment requires temporary occlusion of the blood vessel by special occlusion clamps or pneumatic devices.[11, 13, 14]

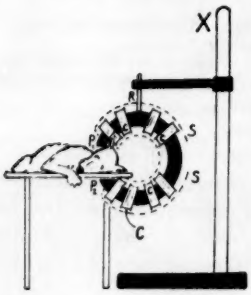

FIG. 2.—External magnet used in conjunction with imbedded sleeve of type shown in Fig. 1*D*. P_1 and P_2, magnet pole pieces; *C*, magnet coils; *S*, grounded shield. (From A. Kolin, *Rev. Sci. Instr.*, **23**, 235, 1952.)

In the initial experiments[4–11] the blood vessel, usually an artery, had to remain exposed during the measurement of blood flow. Thus anesthesia had to be maintained. A step toward elimination of anesthesia was taken by implanting the lucite sleeves containing the pickup electrodes into the animal, leaving the blood vessel in its normal place.[13, 14] In addition, a way was found to establish a zero base line without occluding the blood vessel to stop the blood flow.[15] This was accomplished by means of pickup sleeves with four electrodes. After the implantation of the pickup unit, the animal was placed in the gap of a large electromagnet (Fig. 2), and the flow was recorded as in previous modifications of the method.[14, 16]

The drawback of the above modification of the method lies in the necessity of maintaining the animal immobile in the magnetic field to avoid unbalancing the zero adjustment. The possibility of such a disturbance is avoided in the modification presented in this paper. This is achieved by a rigid coupling between the magnet, the electrodes, and the blood vessel. This coupling is accomplished by using a miniature magnet unit (Figs. 3*A* [front view] and 3*B* [side view]) with a pickup sleeve (*S*) fixed in its gap. This entire unit is implanted into the animal, the blood vessel under study being inserted into the pickup sleeve channel through the slit *Sl* (Fig. 3*C*).

2. *Design of the Miniature Flowmeter Implant.*—Figure 3*C* shows a photograph of a magnet used for flow measurement in the carotid artery of a dog. The lucite sleeve *S* is inserted in the gap of the magnet. It was found most practical to use miniature magnets manufactured for erase heads and recording heads of magnetic

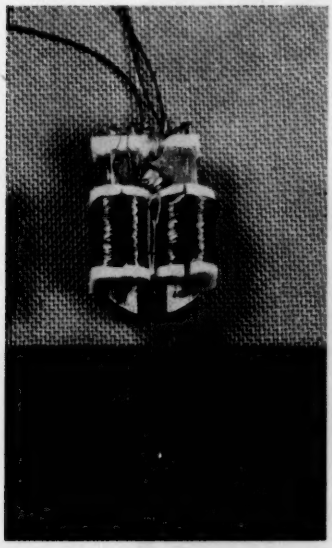

FIG. 3A.—Front view of magnet

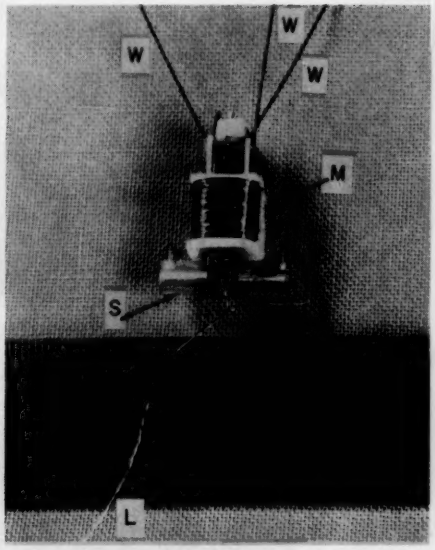

FIG. 3B.—Side view of magnet M and sleeve S. W, suspension wires; L, electrode lead wires (scale in inches).

tape recorders. The magnet shown is from a Brush Electronics Company magnetic-tape erase head, Model BK944. Its dimensions are indicated by the inch scale. Its weight was 5.5 gm. The coils had to be rewound on a nylon or teflon bobbin, since the commercially provided one dissolves in the monomer used in the polymerization process (see below). The total number of turns of both coils (#39 formvar insulated copper magnet wire) was 1,600, and the current used in continuous operation was 65 milliamperes, which resulted in a magnetic field of 800 oersteds in the gap. The electrodes were made of $^3/_4$-mm.-diameter brass rod. The tips of the brass electrodes, flush with the inner surface of the sleeve S, were gold-plated after the completion of the flowmeter unit. Figure 4 shows the sleeve S in the gap of the magnet M (greatly magnified). P_1 and P_2 are the magnet pole pieces, E_1 and E_2 are the electrodes, Sl is the slit, and W_1 and W_2 are the lead wires connecting the electrodes to the amplifier. W_1 has been shown for clarity inside the sleeve body, but actually it runs through a shallow groove in the lucite which is located between the sleeve and the pole piece P_1 coplanar with the line joining E_1 to E_2. The two wires W_1 and W_2 are twisted to minimize the emf induced in the input leads.

In order to secure adequate electrical insulation between the magnet wire, the electrode leads, and the animal, the combination of magnet and sleeve is cast into a block of plastic (methyl methacrylate). The limits of the plastic are indicated by dashed lines in Figure 4. The magnet lead wires and the electrode leads are insulated by separate flexible polyvinyl tubes ($^3/_{32}$-inch i.d.; $^1/_8$-inch o.d.) through which they run (T_1 and T_2). A ground wire connected to the laminated magnet core made of sheets of silicon steel ($^3/_8$ mm. thick) is imbedded in the same tubing as the magnet leads. The polyvinyl tubing is polymerized within the methyl meth-

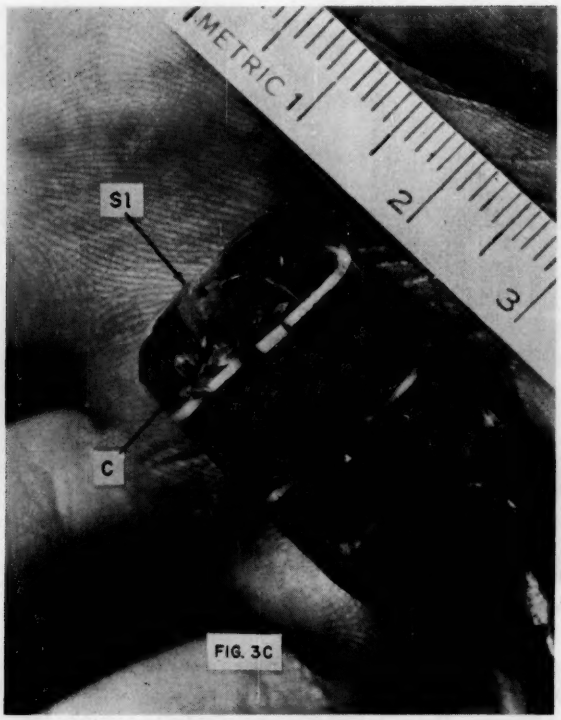

FIG. 3C.—View of flowmeter encapsulated in pastic. *SI*, slit; *C*, artery channel.

acrylate plastic, so that a very secure watertight seal is achieved. The adequate moistureproof electrical insulation between the above-mentioned components of the system was the main experimental difficulty which had to be overcome. Miniature magnets, small enough to be inserted into the neck of a cat, were prepared by one of the authors (A. K.) in 1952 (unpublished), and successful tracings of blood flow in the cat's carotid artery have been obtained. But the flowmeter units were of limited value, owing to an inadequate seal between the polyethylene tubing which was used to insulate the leads and the insulating material encapsulating the magnet. The present method of insulation results in flowmeter units which remain in perfect condition even after prolonged implantation into an animal.

Figure 5 illustrates the casting procedure. The magnet M (seen in side view) is suspended with the gap (space behind P_1) facing downward in a glass vial V. The lucite sleeve S, with the pickup electrode E_1 showing at the bottom, is inserted in the gap. The polyvinyl tubing (T_1 and T_2) insulating the lead wires dips into the vial. The methyl methacrylate is then poured into the vial. The vial is now immersed for 6–8 hours in a constant-temperature bath kept at 120° F. One milligram of benzol peroxide is then added per cubic centimeter of monomer, to act as an accelerator for the polymerization process. The casting is accomplished by polymerizing several layers of the monomer successively. Upon completion of the polymeriza-

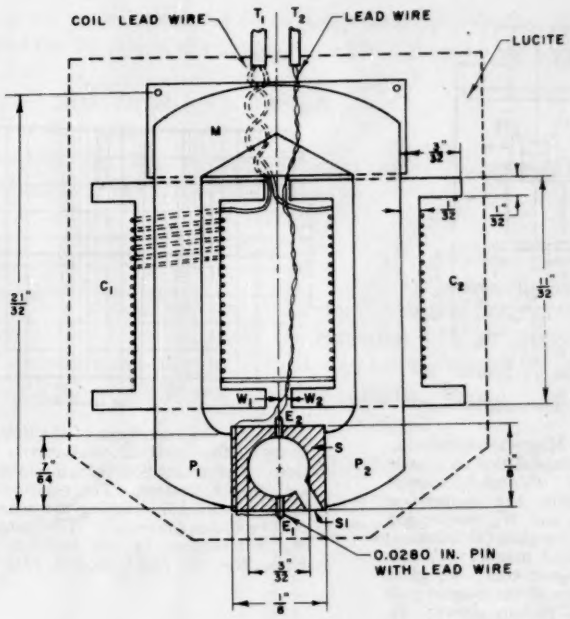

FIG. 4.—Front view of electromagnetic flowmeter. T_1 and T_2, insulating polyvinyl tubing; M, magnet core; C_1 and C_2, magnet coils; W_1 and W_2, electrode lead wires; E_1 and E_2, pickup electrodes; P_1 and P_2, pole pieces of magnet; S, pickup sleeve; Sl, slit.

tion process, the glass vial is broken and excess lucite (polymethyl methacrylate) is removed by abrasive action of a wet sanding-machine belt.

Prior to the polymerization, the cylindrical channel of the sleeve S (Figs. 4 and 5) is filled with a low-melting-point metal alloy (Cerrolow 136). This secures the alignment of the electrode pin (a $^3/_4$-mm. brass rod of which E_1 and E_2 are initially the ends) during the process of polymerization, during which the lucite sleeve, which supports the electrodes, may dissolve. After solidification of the plastic, the Cerrolow core is drilled out, and with it the portion of the brass rod which lies between E_1 and E_2. The surfaces of the electrodes E_1 and E_2 thus produced are flush with the inside wall of the sleeve. The last step consists in gold-plating the electrode tips electrolytically. After removal of all sharp edges and polishing, the flowmeter unit is ready for implantation. A more detailed description of the manufacturing process of the flowmeter units will be published elsewhere.

3. *Calibration and Response to Pulsating Flow.*—The emf (in volts) induced in a conducting liquid flowing through a pipe at right angles to a magnetic field is given by the expression

$$V = 10^{-8}\,\mu H d\bar{v}, \qquad (1)$$

where μ is the permeability, H is the magnetic field strength in oersteds, d is the

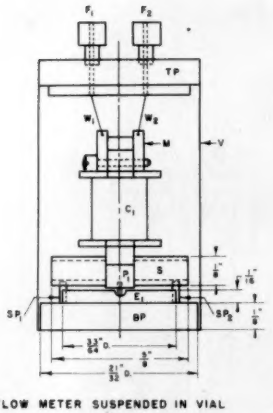

FIG. 5.—Magnet suspended in vial for encapsulation in plastic (side view). F_1 and F_2, clamps for fastening the suspension wires; W_1 and W_2, suspension wires; TP, top plate (aluminum); M, suspended magnet; C_1, one of the magnet coils; V, glass vial; P_1, one of the magnet pole pieces; S, pickup sleeve; E_1, protruding pickup electrode; BP, base plate (aluminum); SP_1 and SP_2, support pins.

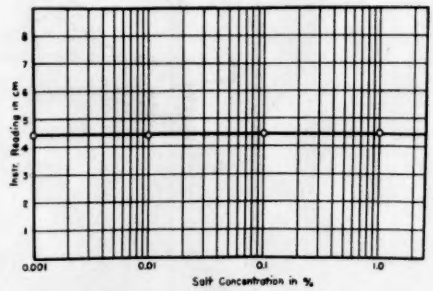

FIG. 6.—Independence of the flowmeter sensitivity of the electrical conductivity of the solution. A constant flow is maintained through an a.c. pipe flowmeter. The conductivity of the liquid is varied by varying the concentration of NaCl solution (*abscissa*). The instrument reading is measured by the ordinate. (From A. Kolin, *Rev. Sci. Instr.*, **16**, 109, 1945.)

diameter of the pipe in centimeters, and $\bar{v}$ is the average velocity of the liquid in centimeters per second.[4] This emf is independent of the electrical conductivity of the liquid (Fig. 6, reproduced from an earlier paper[16]) as well as of the velocity distribution, provided that the latter is axially symmetrical.[16] Contrary to intuitive expectation, the emf induced in the moving fluid for a given volume rate of flow is the same regardless of whether the fluid flows through a nonconducting pipe or through an artery whose electrical conductivity is nearly the same as that of the fluid. This is true regardless of the thickness of the artery wall.[14] This paradox is a consequence of the independence of the induced emf of the velocity distribution, as pointed out in an earlier paper.[14] It is thus unnecessary to calibrate the instrument with each individual artery. One single calibration, once performed without an artery, standardizes the instrument. These results do not depend on the character of the magnetic field used. They are valid for constant as well as for alternating fields; for sinusoidal as well as for nonsinusoidal ones, such as the square wave shape used by Dennison, Spencer, and Green.[17]

The linearity of the calibration is apparent from equation (1) and is verified by experimental results using an excised carotid artery (Fig. 7*A*, reproduced from a previous paper[14]). The same result is obtained when calibration is carried out with a pulsating flow *in vivo* (Fig. 7*B*). The instantaneity of the response of the flowmeter to variations in flow has been confirmed by Inouye and Kuga[18] through careful experimental studies.

It is sufficient to establish the calibration for one single size of pipe. The instru-

ment sensitivity for other diameters follows easily from the following consideration: The emf induced for the rate of discharge $Q = \pi R^2 \bar{v}$ is given by the equation

$$V = 10^{-8}\,\mu H(2R) \cdot \bar{v} = 10^{-8}\,\mu H\,\frac{2R \cdot Q}{\pi R^2} = \frac{10^{-8}\,2\mu HQ}{\pi R} \qquad (2)$$

V is thus inversely proportional to R at a constant rate of discharge Q. The sensitivity $S = V/Q$ can thus be expressed as follows:

$$S = \frac{K}{R} \qquad (3)$$

where $K = 10^{-8}\,(2\mu H/\pi)$ is an instrument constant. Having determined S for one particular diameter, we have calibrated the instrument for all artery diameters. In the case of the miniature-magnet method, one has the choice of perfusing the sleeve channel with saline at a known rate prior to cutting the slit *Sl* or of inserting

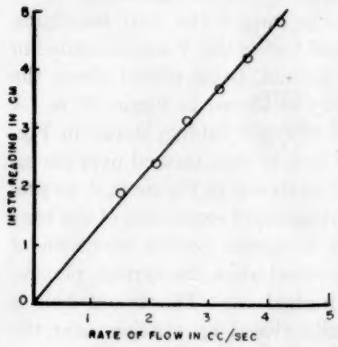

FIG. 7*A*.—Calibration curve for a.c. blood flowmeter. An excised carotid artery is perfused with blood at constant rates. (From A. Kolin, *Rev. Sci. Instr.*, **23**, 235, 1952.)

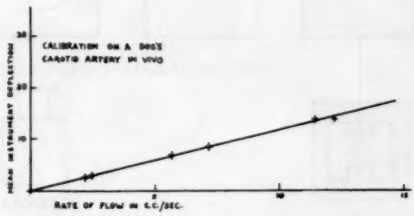

FIG. 7*B*.—Calibration is carried out with a pulsating blood flow through a dog's carotid artery *in vivo*. A constant-field flowmeter is used. (From A. Kolin, *Am. J. Physiol.*, **122**, 797, 1938.)

an excised artery through the slit and perfusing it with blood. Practically, the perfusion consists of ejection of a known volume of saline or blood through the flowmeter unit from a syringe while recording the flow signal. The record thus obtained is shown in Figure 10, *B*, *c*. The injection time is given by the extent of the record along the horizontal time axis. The ratio of the injected volume to this time is the average flow. The average-flow signal is obtained by planimetry of the record of the instantaneous flow and division of the area obtained by the horizontal extent of the curve. The ratio of the average-flow signal thus obtained to the average flow gives the instrument sensitivity in terms of the instrument reading in centimeters per unit flow of 1 cc/sec. In the experiments described below, the sensitivity was 4 mm. per cc/sec, but higher values can easily be obtained by increasing the amplification.

4. *Apparatus.*—The amplifying and recording apparatus used was essentially the same as described in a previous paper.[14] The magnet was energized by a 60-cycle alternating current. Figure 8 shows the block diagram of the apparatus. The signal voltage derived from the flowmeter *FM* is put in series with the output of the

compensator (*comp.*). The compensator voltage can be varied independently in phase and in amplitude,[14] by one single control knob. This permits rapid compensation of stray 60-cycle pickup in the input circuit which may mask the flow signal to be recorded. This compensation must be accomplished at zero flow. In order to be able to stop the flow through the artery, a pneumatic device was used. It is an improvement upon the occlusion bags described earlier.[14] Figure 9 shows the occlusion unit and its components. A tuning-fork-shaped form figure (*a*) is prepared from a low-melting-point alloy (Wood's metal). By repeated dipping of this form in a solution of polyvinyl in a suitable solvent ("Cadco 201"), a sufficiently strong skin of polyvinyl sheeting is formed on the surface of the metal after the evaporation of the solvent. The Y-shaped form is then dipped into water at 100° C. The metal form melts and flows out of the polyvinyl bag. The open central "tail" of the Y is cemented by polyvinyl cement (solution of polyvinyl in "Cadco 201") to a long polyvinyl tubing which is attached to a syringe, while the two remaining closed legs of the Y are imbedded in the animal, being placed about the artery as shown in Figure 9, *b*. A hull of tygon tubing, shown in Figure 9, *c*, is then pushed over the insert as shown in Figure 9, *d*, to prevent outward expansion of the bags and to insure perfect occlusion of the vessel when the syringe plunger is pushed in. The tygon hull is finally closed by stitches near the end of the split section. Figure 10 illustrates the effectiveness of this occlusion device. Short base lines, like those shown in Figure 10, are obtained frequently throughout the measurements, so that a continuous base line can be obtained by joining them. It is during such occlusion periods that the base line is occasionally rapidly adjusted to zero. Once the adjustment is made, readjustment is rarely required.

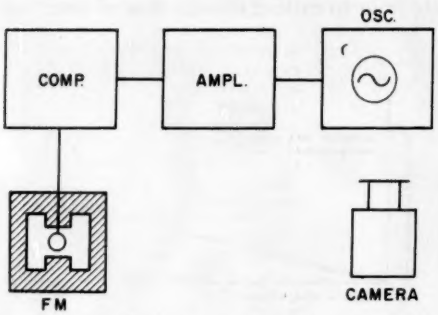

FIG. 8.—Block diagram of the flowmeter circuit. *FM*, the flowmeter transducer; *comp.*, compensator; *ampl.*, amplifier; *osc.*, oscilloscope.

The combined signals of the flowmeter and of the compensator are conveyed in series to the amplifier (RC-coupled single-ended 3-stage) input. The output signal of the amplifier is applied to the oscilloscope (*osc.*) (Dumont type 304), and the oscilloscope reading is recorded by a continuous automatically developing camera, as described in a previous paper.[14]

5. *Examples of Determination of Blood Flow in Unanesthetized Animals.*—Figures 10–12 show records of blood flow obtained with an implanted miniature flowmeter unit of the type described above. These records are not primarily intended to demonstrate physiological results but, rather, are presented as an illustration, obtained with one particular animal, of the possibilities of application of the new modification of the electromagnetic blood flowmeter.

The experiments presented below were obtained with a mongrel dog (weighing 14 kg.) two days after the implantation of the measuring unit. The dog was unanesthetized and was strapped in standing position to avoid moving too far from the

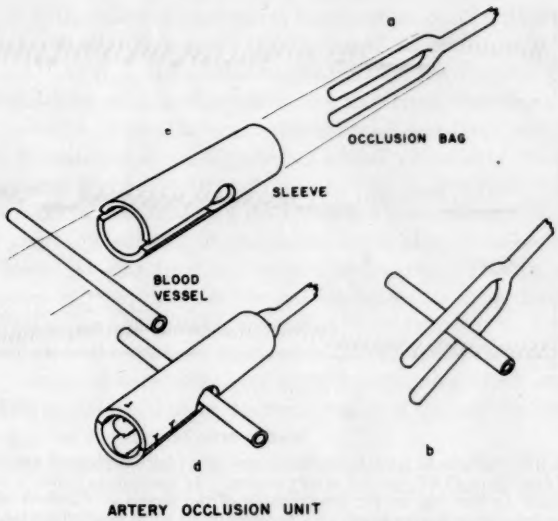

FIG. 9.—Components of the occlusion bag in relation to the artery

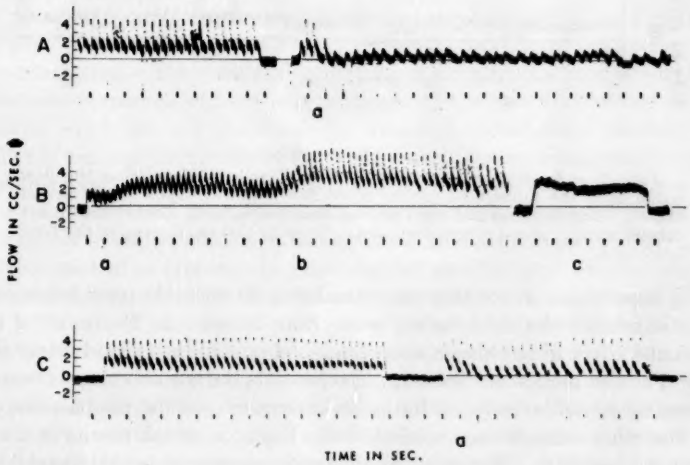

FIG. 10.—Effect of ether anesthesia upon blood flow in dog's left carotid artery and effect of inhalation of smoke. *A*, onset of ether anesthesia (mask applied at *a*). *B*, recovery from ether anesthesia (mask removed at *a*; dog regains consciousness at *b*; *c*, calibration record). *C*, effect of inhalation of smoke upon carotid blood flow; inhalation begins at *a*. The base line is obtained by pneumatic occlusion of the artery.

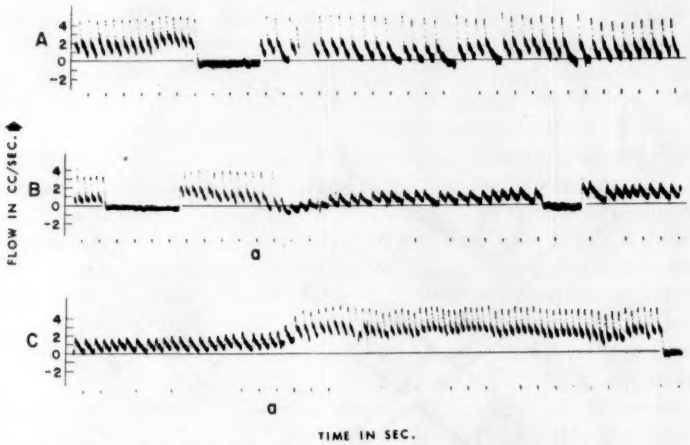

FIG. 11.—Effects of a visual stimulus and of a pharmacological agent upon blood flow through left carotid artery in dog. *A*, reaction to flame (a torch is presented to the dog at the interruption of the record). *B*, effect of amyl nitrite (inhalation begins at *a*). *C*, recovery from effect of amyl nitrite. The inhalation ends at *a*.

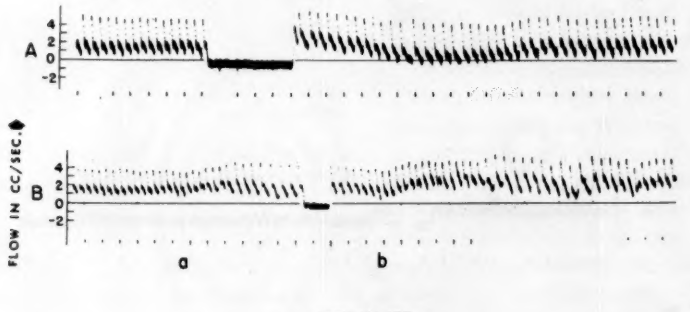

FIG. 12.—*A*, effect of occlusion of carotid artery interrupting the flow signal upon the subsequent carotid blood flow. Record taken in left carotid artery. The other carotid was tied. *B*, reaction to food. Blood flow in left carotid artery. Food is presented at *a*. Chewing and swallowing of the food begin at *b*.

recording apparatus. Blood flow was recorded in all the subsequent tracings in the left carotid artery; the right carotid artery was ligated. In Figure 10, *A* and *B*, the profound effect of anesthesia upon the blood flow in the carotid artery is illustrated. The first section of Figure 10, *A*, represents the normal control tracing. It is followed by a brief occlusion of the carotid artery to establish the base line at zero flow. The ether mask is then applied to the dog at *a*, which results in a marked reduction in blood flow. Figure 10, *B*, shows the increase in carotid blood flow during recovery from ether anesthesia. The mask is removed at *a*. The sharp increase in flow upon regaining consciousness at *b* is noteworthy (*c* is a calibration record). Figure 10, *C*, shows a marked effect of inhalation of cigarette smoke (inhaled at *a*) upon the carotid blood flow.

In Figure 11, *A*, the effect of excitement by presentation of a flame (torch) upon the carotid blood flow is illustrated. In Figure 11, *B* and *C*, a pharmacological effect is depicted. At *B*, *a*, the animal begins inhaling amyl nitrite. The reversal of the normal direction of diastolic flow during the initial diastoles is noteworthy. At *C*, *a*, the inhalation of amyl nitrite is discontinued and the blood flow goes up. In Figure 12 the filmstrip *A* shows the effect of a brief occlusion of the carotid artery upon the subsequent blood flow through it. As can be seen from this record, the initial brief increase in flow following release of the artery is substantial (approximately 45 per cent). Figure 12, *B*, shows the effect of stimulation by food upon carotid blood flow. At *B*, *a*, food is presented to the dog. There is an immediate noticeable increase in flow; at *b*, as the animal begins to chew the food (ground meat), the flow increases and becomes somewhat less regular.

Other types of stimuli have been tried, such as frightening the animal by a light flash. It was interesting to note the very rapid conditioning which occurred. The carotid blood flow was reduced upon the presentation of the light bulb to the animal prior to flashing it for the second time.

These examples illustrate the possibility of studying the effects of physiological and psychological stimuli as well as of pharmacological agents upon essentially normal conscious animals. Chronic research in experimental pathology also appears to be within the realm of possibility. Until now, the implants have not been left in the animal for more than a week, because of thrombosis caused probably by excessive constriction of the artery. There are good reasons to expect that, after elimination of this condition, the implants could be left in the animal indefinitely. The demonstration of the absence of objectionable biological effects of the implants, when properly used, as well as of physical artifacts affecting the measurement of flow, will be presented in a subsequent publication.

The preceding examples demonstrate some of the main advantages of the new modification of the electromagnetic method over the traditional methods of blood-flow determination. The possibility of obtaining a continuous flow record showing instantaneous responses without interference with the flow enables one to detect phenomena which are not observable by means of conventional methods. The possibility of making chronic implantations of sensing elements suggests the applicability of this method to research in experimental pathology and therapeutics. The use of conscious animals promises pharmacological results of greater validity than those obtained in earlier studies on anesthetized animals and permits the application of this method to problems in physiological psychology. Finally, the possibility of implanting miniature flowmeters simultaneously about several different arteries will make it possible to study the correlation between the functions of several different organs and their simultaneous response to a variety of stimuli.

The next step in this work will consist of improving the apparatus and procedure so as to make it adaptable to the measurement of blood flow in human beings and in animals in any accessible blood vessel of sufficiently large diameter. It will be possible to measure blood flow in arteries $^1/_2$ mm. in diameter. This is not necessarily the lowest attainable limit.

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ON THE BINDING OF ADENOSINE TRIPHOSPHATE BY ACTOMYOSIN

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Several attempts[1–4] have been made to study the combination between adenosine triphosphate (ATP) and actomyosin quantitatively, by determination of the viscosimetrically or turbidimetrically measurable change induced in the protein by its combination with ATP. The first efforts [1, 2] were preliminary, in that the available methods did not permit sufficiently rapid measurement of these transient phenomena. But even the measurements performed with a rapid technique[4] are

not quantitative, since, by the time the response has become maximal, an unevaluated fraction of the ATP has been hydrolyzed and replaced by ADP, which inhibits competitively.[5] Hence all previous estimates of the quantity of actomyosin reacting with 1 mole of ATP are too low.

A new method of determining the stoichiometry of this interaction is provided by the experiments of Mommaerts and Hanson,[6] who measured the turbidity change $\Delta\tau$ caused by small amounts of ATP maintained by a rephosphorylating enzyme (pyruvate kinase) and a reservoir substrate (phosphoenol pyruvate), at pH 6.9, 0.01 M $MgCl_2$, ionic strength about 0.5 (mainly KCl). From the ATP-actomyosin association curve so obtained (Mommaerts and Hanson,[6] Fig. 6), we shall derive the total number n of moles of ATP bound by 10^6 grams of actomyosin necessary to produce the total physical change, $\Delta\tau_{max}$, and, furthermore, calculate the intrinsic association constant, k, of this combination reaction.

It is assumed that the relative turbidity drop is proportional to the amount of bound, ATP, r being the bound ATP in moles per 10^6 grams of actomyosin:

$$\frac{r}{n} = \alpha = \frac{\Delta\tau}{\Delta\tau_{max}}.$$

The concentration of bound ATP is $r(M)\cdot 10^{-6}$ moles per liter for an actomyosin concentration of (M) grams per liter and becomes $n(M)\cdot 10^{-6} = x$ for the case of maximal response. If c' is the concentration of free ATP, and c that of the total ATP, we can write[7]

$$\frac{1}{r} = \frac{1}{nkc'} + \frac{1}{n}$$

and hence

$$k = \frac{r}{(n - r)c'} = \frac{r/n}{(1 - r/n)(c - rx/n)} = \frac{\alpha}{(1 - \alpha)(c - \alpha x)}.$$

This is valid for any combination of points i, j, on the association curve, and x is obtained from

$$\frac{\alpha_i}{(1 - \alpha_i)(c_i - \alpha_i x)} = \frac{\alpha_j}{(1 - \alpha_j)(c_j - \alpha_j x)}.$$

In this manner we obtain $n = 1.98 \pm 0.12$ moles per 10^6 grams, so that 1 mole of ATP is bound per 504,000 grams of actomyosin. This equivalent of protein may still be somewhat underestimated, since it has been implied that all the nucleotide is maintained in the form of ATP. This condition was aimed at in the experiments by the use of a large excess of pyruvate kinase, but it is unknown as yet to what extent this was successful. Any imperfection in this regard would tend to reduce c below the accepted values and hence to increase the equivalent weight of the protein. We decline, for the moment, to interpret the result in terms of the molecular weight of myosin, in view of the uncertainty of the latter.

Further, we find $k = (6.7 \pm 0.15)\cdot 10^6$, and hence $\Delta F^\circ_{200} = RT \ln k = 9{,}450$ cal. The fact that a constant value of k applies throughout the curve corroborates the underlying assumption that $\Delta\tau$ is proportional to α, unless there chances to be a

compensation of errors due to this factor and to differences in the completeness by which c is maintained by rephosphorylation at different ATP levels. It is of importance to emphasize the high value of $\Delta F°$ which, having been estimated before,[2, 3] is now obtained with more satisfactory precision and is found to exceed even the $\Delta F°$ and ΔH values of the hydrolysis of ATP.[8–10] It should be kept in mind that the value of k, notwithstanding its seemingly different mode of determination, is still essentially identical with a Michaelis-Menten constant. Hence it is a true association constant only if the velocity constants for combination and dissociation are considerably greater than that for the enzymatic decomposition. If this were not the case, k and $\Delta F°$ would have been underestimated.

Considering the excess of Mg over ATP in these experiments, it should be kept in mind that practically all ATP is in the form of the Mg complex[11, 12], so that the above derivation applies to this rather than to ATP as such.

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*RESULTS OF INVERSION OF NEURAL PLATE MATERIAL**

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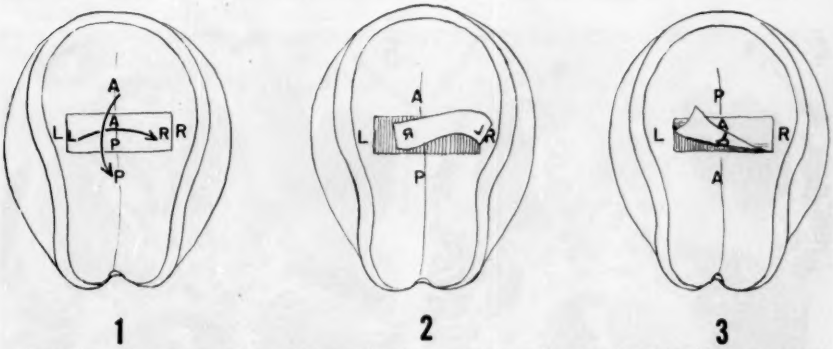
Capacities for differentiation have frequently been tested by transplanting or explanting parts of the embryonic nervous system. Experiments of this nature have contributed much information concerning the time of tissue determination, of regional determination, and specificity.[1–3] In the present investigation the ability of the medullary plate to form nervous system under varied conditions of autotransplantation has been studied.

It is well known that the chordamesoderm influences the overlying ectoderm to form nervous system. The present experiments were made to study development of the nervous system under conditions of inversion. A strip of medullary plate together with underlying archenteric roof was cut out of neurulae of the spotted

salamander *Amblystoma punctatum* and replaced in all possible varying axial relations. The following questions were asked: (1) How do these reversals affect the nervous system and the mesodermal derivatives? (2) Will the structures formed adjust themselves to the level of the embryo as a whole? (3) Will the reimplanted structures be otherwise adjusted to their surroundings?

The operations performed are diagramed in Figures 1–3. Axes of the strips were reversed as follows: anteroposterior and mesiolateral (Fig. 1); mesiolateral and dorsoventral (Fig. 2); and anteroposterior and dorsoventral (Fig. 3). Stages used varied from Harrison's Stage 14 to Stage 16. The level of the strip was varied either anteriorly or posteriorly in the embryo to test its regional specificity.

Healing in all series occurred remarkably rapidly. The graft maintained its shape. In the operations involving dorsoventral reversal the graft remained sharply outlined because of the contrast between the lightly pigmented chordamesoderm and the fully pigmented neural plate surrounding it.



FIGS. 1–3.—Diagrams of the early neurula of *A. punctatum*, showing the field of operation and indicating the orientation of the strip of the prospective neural plate which was modified experimentally in its axial relations. Fig. 1, anteroposterior and mesiolateral axes reversed. Fig. 2, mesiolateral and dorsoventral axes reversed. Fig. 3, anteroposterior and dorsoventral axes reversed.

In the most favorable cases in the first series (as in Fig. 1), reversal without inversion, the closing of the neural folds was not retarded. The folds arched over the graft and obscured it from view. Subsequent development was fairly normal during the early stages, except for a thickening of the neural tube in the vicinity of the region of operation. As development progressed, the larvae were seen to have shortened bodies; it was suspected that this was due to defective development of the notochord, a suspicion later justified by study of the sections.

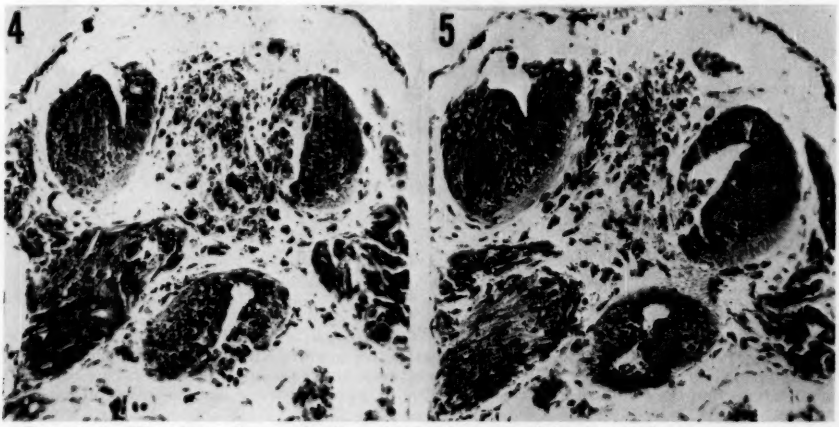
The nervous system in the first series (Fig. 1) has always developed regularly, irrespective of the level of operation. Marked deficits, however, have been found in the principal axial mesodermal structure, the notochord. In the less favorable cases, particularly those performed in the late neurula, the irregularities were chiefly those of blockage and malformation of the posterior head and trunk. The chordamesoderm evidently served as a partial block to one or the other of the enveloping neural folds.

In the second and third series (Figs. 2 and 3), where the dorsoventral axis was

reversed, an entirely different course of development resulted. The nervous system from the graft was inverted. The only animals in which the transplanted nervous system showed any regularity were among the group operated at Stage 14. The inverted strip acted as a block to the nerve tube of the host; at the level of the graft it can be seen in sections that three nerve tubes are present (Figs. 4 and 5). Two lateral ones derive from the host medullary folds, a median one from the graft. In the case of the series (Fig. 2) in which the anteroposterior axis of the transplant was not reversed, the nervous structures induced in the graft proved to be of the appropriate level, as would be expected.

The chordamesoderm in inverted position is in most cases prevented from attaining its characteristic organization. It forms dissonant elements of unique cellular structure, as shown in Figures 4 and 5. In the third series, where the anteroposterior axis was reversed, the notochord appeared to form a more extensive indeterminate mass than in the second.

The chordamesoderm had already gastrulated normally before the operation and



FIGS. 4, 5.—The photomicrographs show the development of three typical nervous systems resulting from the operation shown in Fig. 3. The median nervous system has developed from the grafted strip; the two lateral ones, from regularly developing neural folds which were separated by cordamesoderm during the period of closing of the neural folds. The inchoate mass between the three nervous systems is composed of chordamesoderm which forms an indeterminate cellular mass lacking characteristic notochordal structure.

had performed its primary induction. Marx,[4] in Spemann's[5] laboratory, had shown that, under other conditions of transplantation, the notochord-forming region was the one region of the early gastrula able to realize its own potentialities. The most striking result of the present experiment has been to show that at a later stage, after the neural plate and the archenteric roof have started on the course of their differentiation, the notochord is far more dependent on its surroundings than is the nervous system.

Thus, in answer to the questions posed above, the following would appear to be true: (1) The nervous system maintains its identity under the various sorts of reversal imposed. The notochord fares less well. Even if left beneath the ectoderm, reversal of anteroposterior and transverse axes appears to disturb its elongation and

its integrity. If differentiating notochord is placed dorsal to the medullary plate, it evidently ceases to develop as a structural entity, although cellular differentiation progresses. (2) The nervous system at the time of operation is capable of regulating, with regard to level, reversals of the anteroposterior and transverse axes but not reversals of the dorsoventral and anteroposterior axes imposed simultaneously. The notochord seems not to regulate anteroposterior reversal. (3) The archenteric roof and medullary plate retain their respective identities under conditions of reversal and do not adjust to their new position when the dorsoventral axis is reversed. In such a graft the chordamesoderm acts as an intrusive developmental block and separates locally the developing neural folds each of which forms a complete reduced nervous system characteristic of its original localization.

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